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**Host Resistance and Viral Transcription as
Determinants of MMTV Tumorigenesis**

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**Host Resistance and Viral Transcription as
Determinants of MMTV Tumorigenesis**

by

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To my family

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Host Resistance and Viral Transcription as Determinants of MMTV Tumorigenesis

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Mouse mammary tumor virus (MMTV) is an oncogenic retrovirus that causes mammary tumors in susceptible mice within 6 to 9 months of infection. Type B leukemogenic virus (TBLV) is a variant of MMTV that induces thymic lymphomas in mice, instead of mammary tumors, with a shorter latency. Exogenous MMTV has a complicated life cycle involving milk-borne virus transmission and tissue type and differentiation-specific regulation of viral transcription. Most inbred mouse strains also harbor endogenous MMTV proviruses (*Mtvs*) in their germline that are inherited according to Mendelian rules and are usually not associated with

mammary tumorigenesis.

This thesis reports studies on the intricate relationship of MMTV with its host by demonstrating that the outcome of MMTV infection is influenced by both the composition of viral transcriptional elements and by host genetics. This is the first demonstration that the absence of negative regulation of viral transcription and presence of a T-cell specific enhancer in lymphoid cells converts a mammotropic MMTV to a lymphotropic virus. Interestingly, the endogenous *Mtv* loci in the BALB/cJ mouse genome are required for susceptibility to infection and tumorigenesis by both MMTV and TBLV. A BALB/cJ congenic mouse strain lacking endogenous *Mtvs* (BALB/*Mtv*-null) is highly resistant to both MMTV and TBLV. The resistance is not mediated by the production of MMTV-specific neutralizing antibodies in the BALB/*Mtv*-null strain. However, cell-mediated immune response directed against MMTV-infected cells might contribute towards the tumor resistance phenotype. The BALB/*Mtv*-null mice also are resistant to oral infection with the bacterial pathogen *Vibrio cholerae* compared to BALB/cJ mice, and this resistance to *V. cholerae* correlates with the absence of endogenous *Mtvs*. Thus, the BALB/*Mtv*-null mice demonstrate a novel mode of resistance to multiple pathogens that appears to be linked to expression of MMTV genes. These data support a role for endogenous *Mtvs* in determining susceptibility to multiple pathogens.

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Introduction

1.1 History

Mammary tumors were first described in wild mice in 1854 [89]. In 1918, Lathrop and Loeb demonstrated a genetic influence on the development of mouse mammary cancer [239]. Subsequently, a non-chromosomal influence on the etiology of mammary tumors in mice was detected [210]. Sixteen years later, Bittner associated this non-chromosomal influence to nursing on females from high-tumor incidence strains [44] and, alternately, elucidated the role of inherited susceptibility, and hormones on the development of spontaneous mammary tumors in mice [45]. A transmissible milk influence was characterized as a high molecular weight colloid and suggested to be a virus in 1942 [447]. Subsequently, spherical virus-like particles with a dense central core were observed in the mammary carcinomas that developed in high-tumor-incidence mouse strains [346]. In the following year, virus-like particles purified from the milk of a high-tumor incidence mouse strain were shown to induce mammary carcinomas upon inoculation into a low-tumor-incidence mouse strain [168].

Several studies established that all tumors from high-tumor-incidence mouse

strains contained two kinds of virus-like particles, intracytoplasmic particles characterized by a concentric double membrane (type A) and extracellular particles characterized by an eccentrically placed nucleoid (type B) [37]. The type-A particles were proposed to transform into B-type particles following passage through the cell membrane [37]. B-type particles purified from milk and mammary tumor tissue were demonstrated to have a nucleoprotein composition containing RNA [257, 258]. The particles were ether-sensitive and contained radially oriented outer membrane spines [257]. In 1963, Moore proposed that these virus-like particles represented Bittner's milk agent [301]. He also suggested that the milk agent could become heritable. Subsequently, reverse transcriptase (RT), the hallmark DNA polymerase of retroviruses, was detected in the milk-borne mouse mammary tumor virus (MMTV) [391, 392, 396]. Thus, MMTV was the first identified mammalian retrovirus [448].

1.2 Classification

MMTV belongs to the virus family *Retroviridae* whose members are enveloped, spherical RNA viruses that undergo reverse transcription during their replication cycle [63]. This family is comprised of two subfamilies – (i) *Orthoretrovirinae* which includes six genera [*Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, and *Lentivirus*] and (ii) *Spumaretrovirinae*, which has a single genus *Spumavirus*. MMTV is currently assigned to the genus *Betaretrovirus* in the subfamily *Orthoretrovirinae* [63].

1.3 MMTV virion morphology

MMTV virions have capsids with an average diameter ranging from 130 to 140 nm and icosahedral symmetry [58, 384]. The eccentrically positioned nucleocapsid core [37] is irregularly sized and shaped and is often angular [58]. The dimeric viral genome is comprised of non-segmented, plus-sense, single-stranded RNA (2% by weight) [63, 114]. Proteins and carbohydrates constitute approximately 60% and 3% of the virion weight, respectively, [63] and lipids derived from host cell membranes constitute up to 35% of the virion by weight [63].

The viral membrane displays approximately 400 prominent glycoprotein spikes that project radially from the viral membrane surface [58]. The spikes are spaced approximately 12 nm apart and each spike is surrounded by 5 or 6 immediate neighbors and assume the symmetry of the underlying capsid [58, 353, 384]. The 8 nm long glycoprotein spikes [58] have two distinguishable components – (i) knobs at the distal ends measuring 54.4 ± 10.3 Å in diameter and composed of three subunits 15 to 25 Å in diameter [384], and (ii) thin stalks that connect the knobs to the viral membrane [384].

1.4 MMTV genome organization

The 70 S MMTV genome [114] is composed of two identical genomic RNA molecules joined by non-covalent interactions at the dimer linkage sequence (DLS) near the 5'-end of the genome and facilitated by multiple interactions throughout the

genome [448]. According to the loop-loop kissing complex model, dimerization is initiated by symmetrical intermolecular bonds to form a kissing-loop complex that is subsequently stabilized by formation of an extended duplex [330].

The 35 S monomeric RNA is 8600 nt long with a 15 nt direct repeat (R) at each end. The 5'-end of the genomic RNA has a methylated nucleotide cap with a type 1 sequence of m⁷G5ppp5'GmpNp while the 3'-end is polyadenylated. The R region at the 5'-end is sequentially followed by the 110 nt unique region (U5), the primer binding site (PBS), three out-of-phase, overlapping reading frames encoding the *gag* (group-specific antigen), *pro* (protease), and *pol* (reverse transcriptase and integrase) genes, an overlapping reading frame encoding the *env* and *rem* genes, and the approximately 1200 nt long unique region (U3) that encodes the C-terminal end of the envelope (Env) protein and the out-of-phase, overlapping superantigen (*sag*) gene [209, 300, 448] (Figure 1.2).

Two -1 ribosomal frameshift events during genomic RNA translation allow uninterrupted readthrough from *gag* to *pol* genes, leading to the production of three polyprotein precursors Pr77^{Gag}, Pr110^{Gag-Pro}, and Pr160^{Gag-Pro-Pol} (Figure 1.1) [209, 300]. Translation of Env, regulator of export of MMTV mRNAs (Rem) and Sag requires the synthesis of spliced, subgenomic mRNAs. Therefore, the naked MMTV genomic RNA is not infectious. The genomic RNA presumably harbors one or more encapsidation sequences termed ψ or E that allow the packaging of viral RNA during virion assembly [448].

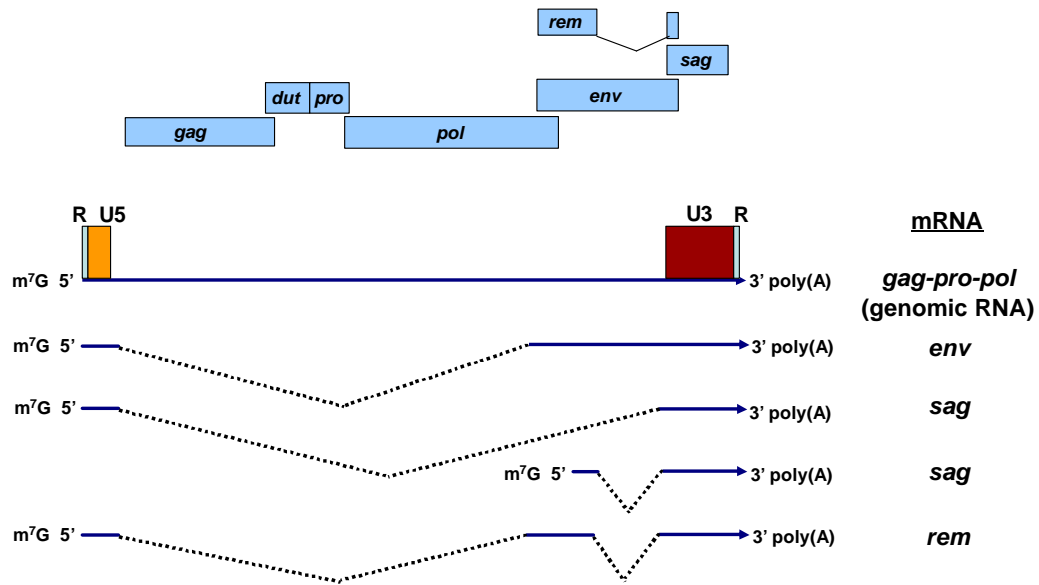


Figure 1.1. Structure of MMTV genomic and subgenomic RNAs.

The full-length genomic RNA encodes the Gag, Pro and Pol proteins while the *env*, *rem* and *sag* coding sequences are interrupted by introns (depicted as dashed V-shaped lines) that are spliced out to generate subgenomic mRNAs.

1.5 MMTV proteins

The 35 S genomic RNA encodes the non-glycosylated structural polyprotein precursors Pr77^{Gag}, Pr110^{Gag-Pro}, and Pr160^{Gag-Pro-Pol} (Figure 1.2) [90, 99, 111, 398]. These large polyprotein precursors are proteolytically processed by the viral protease to yield the proteins found in mature virions. Cellular proteases might also participate in the maturation of viral polyproteins [284].

Two singly-spliced 24 S and 14 S subgenomic mRNAs encode the viral Env glycoprotein and the 36 kDa Sag protein, respectively [101, 111, 398, 400], while a

novel doubly-spliced mRNA encodes the Rem protein (Figure 1.2) [J. Mertz *et. al.*, manuscript submitted].

1.5.1 Group-specific antigen (Gag)

MMTV Gag is cotranslationally directed to the pericentriolar region via its cytoplasmic targeting-retention signal (CTRS) [82, 367] in a microtubule-dependent process through interaction with the dynein/dynactin motor complex [402]. The domain structure of MMTV Pr77^{Gag} polyprotein is defined as: NH₂-MA(p10)-pp21-p3-p8-n-CA(p27)-NC(p14)-COOH, where 'n' represents 17 amino acids present in the viral sequence, but not found as a cleavage product among purified virion proteins [99, 195, 275, 399]. The three domains – matrix or membrane associated (MA), capsid (CA), and nucleocapsid (NC) – are common to all retroviruses.

The mature, 10 kDa MA protein is the most hydrophobic Gag protein [272] and harbors an N-terminal glycine residue with an amide-bonded myristoyl group that is essential for virus assembly and budding [195, 395]. MA proteins of some retroviruses do not undergo myristoylation, but are subject to acetylation and phosphorylation [448]. Some MA proteins also have stretches of basic amino acid residues [448]. MA allows the membrane targeting of viral cores and may also interact with Env during budding [67, 448].

The 27 kDa CA protein [195, 395] is the major structural virion protein that forms the shell around the viral ribonucleoprotein [272]. MMTV CA contains a hydrophobic domain and exists in phosphorylated and non-phosphorylated forms

[272, 323, 385]. Phosphorylation occurs in the Pr77^{Gag} polyprotein, which upon cleavage generates phosphorylated p27 as well as the phosphorylated core protein pp21 [195, 352, 395]. MMTV MA, pp21, p3-p8-n, and CA possess weak interaction potentials, which together form a significant secondary force during Gag self-association [480].

The 14 kDa, highly basic NC protein [195, 395] is responsible for genomic RNA binding and dimerization by interacting with the RNA packaging sequence [448, 480] via its two zinc-coordinating Cys-X₂-Cys-X₄-His-X₄-Cys motifs [38, 188, 195, 225]. NC proteins stabilize the RNA genome in mature virions, stimulate reverse transcription by promoting complementary RNA annealing, facilitate viral DNA elongation by reducing RT pauses at stable stem-loop sites, and also stabilize the viral DNA after reverse transcription [448]. NC also provides the major force for Gag multimerization via its interaction or I domain rich in basic residues [448, 480].

1.5.2 Deoxyuridine triphosphatase (dUTPase or DU)

The dUTPase catalyzes the hydrolysis of deoxyuridine triphosphate (dUTP) to deoxyuridine monophosphate (dUMP) and pyrophosphate, thus increasing the fidelity of reverse transcription by preventing the incorporation of dUMP into DNA and also providing a pool of dUMP, the precursor of thymidine nucleotides [36, 448]. The MMTV dUTPase is a *transframe* 30 kDa protein, p30, generated from the Gag-Pro junction by proteolytic processing of the Gag-Pro polyprotein [194]. The N-terminal 95 amino acids of p30 are produced from the C-terminus of NC while the following 154 amino acids, that display dUTPase homology, are derived

from the 5'-end of the *pro* open reading frame (ORF) [194].

The MMTV dUTPase forms enzymatically active trimers that might directly associate with the viral RNA through the NC domain [36]. This NC domain-mediated anchoring might compensate for the low specific activity of the MMTV dUTPase [36, 230]. Furthermore, location of the MMTV *dut* gene in the *pro* region would generate higher amounts of the enzyme compared to non-primate lentiviruses that harbor the *dut* gene in the *pol* region [36, 230]. In addition to its enzymatic activity, the abundant MMTV p30 protein could also play a structural role in the virion [36, 230].

1.5.3 Protease (Pro/PR)

PR belongs to the aspartic proteinase enzyme family that is characterized by two aspartic acid residues, each placed in the highly conserved motif Asp-Thr/Ser-Gly, which coordinates a water molecule used to hydrolyze the target peptide bond [448]. Retroviral PR contains a single Asp-Thr-Gly motif thus, requiring dimerization for activity [285, 448]. PR gets packaged into the MMTV virion as part of the Gag-Pro and Gag-Pro-Pol polyprotein precursors. A drop in the virion pH might lead to the activation of PR, which is maximally active between pH 4 to 6 [285, 448]. PR undergoes autocatalytic cleavage and performs ordered processing from the N-terminus towards the C-terminus of Gag, Gag-Pro, and Gag-Pro-Pol polyprotein precursors [285, 448]. Ordered processing of the virion polyproteins is an essential step in the viral life cycle as it is intimately linked to virion assembly, budding and maturation [448].

1.5.4 Reverse transcriptase (RT)

MMTV RT displays 3 enzymatic activities – RNA and DNA-dependent DNA polymerase activities using RNA or DNA primers and RNase H (RNA-DNA hybrid: ribonucleotide-hydrolase) activity. Each activity requires divalent cations with a preference for Mg^{2+} ions [108, 421]. MMTV RT uses tRNA^{Lys3} that is hydrogen-bonded to the viral genome for priming of minus-strand DNA synthesis [340]. The RNase H activity of RT cleaves phosphodiester bonds in the RNA strand of a RNA-DNA hybrid producing 3'-OH and 5'-PO₄ groups [108]. RT lacks 3'-5' proofreading exonuclease activity, allowing nucleotide misincorporation. Thus, RT plays a significant role in generating retroviral diversity, rapid viral evolution, and adaptation to the environment [448].

MMTV RT is a *transframe* protein containing at its N-terminus 27 amino acids derived from the C-terminus of PR, while the remaining amino acids are derived from the *pol* gene [124, 421]. The DNA polymerase domain is located at the N-terminus, whereas the RNase H domain is located towards the C-terminus [448]. MMTV RT is enzymatically active as a 66 kDa monomer with a pH optimum of 7.5 for the DNA polymerase activity [421]. RT is a slower enzyme compared to cellular DNA polymerases and is not highly processive.

1.5.5 Integrase (IN)

The retroviral IN protein catalyzes viral DNA insertion into host cell DNA. IN is encoded within the 3'-terminal portion of the *pol* gene immediately downstream from RT sequences and, in the same uninterrupted reading frame.

Proteolytic cleavage from the C-terminus of the Gag-Pro-Pol polyprotein generates active IN [448]. Virion incorporation is determined by Gag-Gag interactions within these precursors.

Retroviral integrases range in size from 280 to 450 amino acids in length [448]. A highly conserved zinc-finger-like HHCC (H-X₍₃₋₇₎-H-X₍₂₃₋₃₂₎-C-X₂-C) motif is found near the N-terminus followed by a conserved DD35E (D-X₍₃₉₋₅₈₎-D-X₃₅-E) motif – a universal feature of integrases and transposases [448]. The DD35E domain functions as the catalytic core of the enzyme while the C-terminal variable domain functions in DNA binding [448].

A tetrameric integrase complex bound to viral DNA ends provides the minimal requirements for concerted integration of both viral long terminal repeats (LTRs) [131]. The inverted repeat sequences at the ends of viral DNA containing the CA/TG dinucleotide pair positioned 2 bp from the ends of the linear precursor are crucial for recognition of viral DNA ends by integrase [448]. Integrase also promotes reverse transcription by direct physical interaction with the nucleoprotein-RT complex [467].

1.5.6 Regulator of export of MMTV mRNAs (Rem)

MMTV encodes a protein (Rem) of 301 amino acids, which regulates the nuclear export of unspliced viral mRNAs to the cytoplasm for translation [J. Mertz *et. al.*, manuscript submitted]. The Rem protein consists of the Env leader peptide (98 amino acids), 162 amino acids from the Env surface protein (SU or gp52) and 41 amino acids from the transmembrane protein (TM or gp36). Rem is expressed from

a novel doubly spliced mRNA (Figure 1.1). Software analysis predicted several protein motifs, including sites for glycosylation, myristoylation, amidation, and protein kinase C phosphorylation. Rem also contains two functional, overlapping nuclear localization signals (NLSs), a putative leucine-rich nuclear export signal (NES) as well as an arginine-rich motif (ARM), which is an RNA-binding domain found in human immunodeficiency virus (HIV) regulator of virion expression (Rev)-related proteins [133, 268]. Rem overexpression partially restores Gag expression from a Rem-deficient MMTV mutant deficient in *gag* mRNA export.

The 33 kDa Rem protein is unique because it is two to three times larger than Rev (13 kDa) and other Rev-like RNA export proteins, including human T-cell leukemia virus (HTLV)-1 Rex (27 kDa) [191, 327] and human endogenous retrovirus (HERV)-K Rec (14 kDa) [264, 253, 477] proteins. The extensive C-terminal region of Rem contains few predicted motifs and may function as a negative regulator. Rem binds a Rem-responsive element that encompasses the 3' end of the *env* and the U3 region [J. Mertz *et. al.*, manuscript submitted].

Rem is primarily detected in nuclei within the nucleolus. MMTV Env leader peptide was also reported to localize to the nucleoli of MMTV-derived T-cell lymphomas where it colocalized with the nucleolar protein B23 [196]. Association with B23 has been previously reported for other auxiliary nucleolar retroviral proteins, such as Rev and Rex [196].

1.5.7 Envelope (Env)

MMTV Env is translated in the order N-SU-TM-C [399] from a 24 S [111], 3.6 kb mRNA that splices out the *gag*, *pro*, and *pol* genes (Figure 1.1) [266, 398]. The 5' splice site for the subgenomic RNA is located approximately 288 nt downstream from the 5'-end of the viral RNA at the U3/R border of the 5' LTR [130]. The *env* gene terminates within the LTR and its 3'-end overlaps the 5'-end of the *sag* gene [189].

The non-glycosylated Env precursor, p67^{Env}, is synthesized on the rough endoplasmic reticulum as a type I transmembrane protein [100]. The signal peptide (98 amino acids) is cleaved between Gly and Glu within the sequence Thr-Gly-Glu-Ser-Tyr to generate the p61^{Env} precursor. The precursor is subsequently glycosylated to produce the gPr73^{Env} protein [100, 189], which contains five asparagine (N)-linked oligosaccharides rich in fucose, mannose, galactose, N-acetyl glucosamine, and N-acetyl neuraminic acid [15, 100, 472]. Glycosylation of gPr73^{Env} is essential for proper maturation and cell surface localization of mature Env proteins [140]. The gPr73^{Env} is then trafficked to the *trans*-Golgi where it is proteolytically processed by cellular proteases like furin [448] and subjected to secondary glycosylation followed by transport to the cell surface [88]. Although a minor population of gPr73^{Env} is found at the cell surface, proteolytically processed Env is critical for the membrane fusion event required for viral entry [448]. Proteolytic processing of gPr73^{Env} occurs by removal of the dipeptide Lys-Arg at the sequence Ile-Arg-Ala-Lys-Arg-Phe-Val-Ala and generates the surface (SU) gp52^{Env} and transmembrane (TM) gp36^{Env} proteins [98, 189]. Two of the N-linked

oligosaccharides are located on the 25.5 kDa TM protein, while 3 are located on the 41 kDa SU protein [189, 360].

The TM protein functions as the membrane anchor via its hydrophobic domain (amino acids 523 through 548) and also complexes with the N-terminal Gag protein p10 [353, 448]. TM also allows Env trimerization shortly after translation in the ER [448] to form the 230 kDa envelope spike [353, 384].

The SU protein serves as the anti-receptor for binding the cell surface receptor. Several other functions have also been attributed to MMTV SU, including (i) Sag presentation to T cells, resulting in stimulation of T-cell proliferation and interleukin 2 (IL2) production [160], (ii) enhancing the sensitivity of mouse mammary epithelium towards prolactin (PRL) by increasing the synthesis and membrane recruitment of PRL receptors [48, 49], (iii) facilitating viral infection by activating B cells via interactions with the surface toll-like receptor 4 (TLR4) [359], and (iv) initiation of mammary tumorigenesis mediated by the immunoreceptor tyrosine-based activation motif (ITAM), which triggers early hallmarks of transformation [219].

1.5.8 Superantigen (Sag)

An open reading frame within the MMTV U3 region encodes a 37 kDa protein that functions as a superantigen (Sag) [273]. Sag presentation induces CD4⁺ T cells to produce cytokines like IL2, IL4 and interferon (IFN) γ [320] followed by clonal, apoptotic deletion of these CD4⁺ T cells [1, 39, 80, 109, 129, 205, 333, 452]. While strong Sags like that encoded by *Mtv7* induce

proliferation of cognate T-cells prior to clonal deletion, weak Sags like C3H Sag and GR Sag fail to induce proliferation of reactive T cells or expression of IL2 receptors (IL2R) [320]. Activated CD8+ T cells bearing Sag-specific TCR V β chains also produce IFN γ following Sag stimulation, but Sag-bearing target cells are not lysed [190]. The endogenous *Mtv*-encoded Sags have been extensively studied previously as minor lymphocyte-stimulating (MIs) antigens [117, 127, 143, 349, 463] and have also been associated with both negative and positive selection of thymocytes [57, 77, 250]. Besides its superantigenic function, Sag also has been reported to transactivate the MMTV LTR-driven transcription [441] and to induce transformation [312].

Subgenomic MMTV *sag* mRNAs [442, 459] have been described from 4 different promoters (i) the classical promoter at the U3-R border (P₁₁₉₆ or P₁) [78, 442]; (ii) an internal U3 promoter (P₆₉₈ or P₂) [173]; (iii) a T-cell-specific phorbol ester-inducible promoter in the *env* gene (P₇₂₄₆ or P_{env}) [120, 293, 316, 481]; and (iv) a second, LTR-proximal promoter (P₈₄₉₈ or P_{env2}) [14] within the *env* gene (Figure 1.2). MMTV strain specific differences may affect the choice of *sag* promoter [470]. A 183-bp segment (E_{pol}) in the *pol* gene (nt 5499 to 5681) upregulates *sag* expression in mature B cells [365] while an intragenic enhancer (E_{proB}) (nt 9 to 556 in the 5' LTR or nt 54 to 991 in the 3' LTR) stimulates the expression of MMTV genes, including *sag*, in pro-B cells [364, 365]. Sag expression is reduced by glucocorticoids despite increased transcription from the 5' LTR [427]. The level of *sag* mRNA expression correlates directly with kinetics of self-reactive T-cell loss [31].

Stable cell surface expression and presentation of Sag requires association with major histocompatibility complex (MHC) class II molecules [41, 46, 169]. MHC class II I-E molecules present Sag more efficiently than the MHC class II I-A molecules. The I-A allotypes show a hierarchy of Sag-presentation efficiency, which in turn determines the magnitude of the T-cell response and the extent of help provided to infected B cells [166, 187]. Some MMTV Sags (GR, C3H, SIM, *Mtv1*, 6, and 9) require class II I-E for presentation while others (*Mtv7*, SW) can be presented by both class II I-E and I-A molecules. A diverse repertoire of MHC class II-peptide complexes is also critical for recognition of MMTV Sag by T cells [166], and the amount of cell surface class II limits Sag presentation [254].

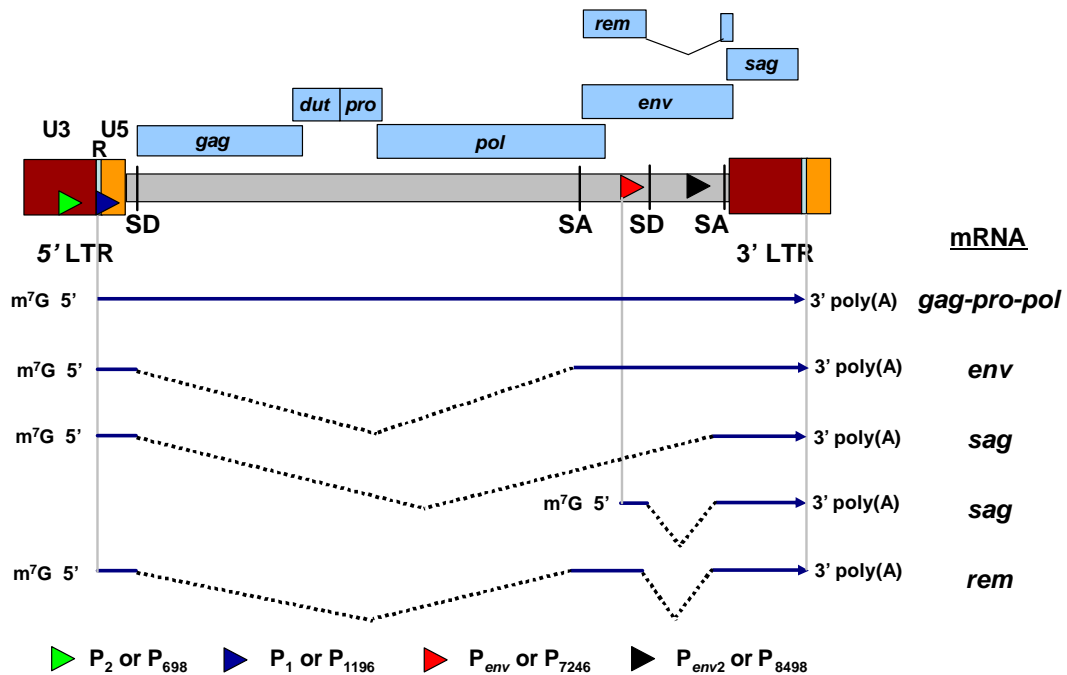


Figure 1.2. Structure of MMTV genome and transcripts.

The mature 47 kDa Sag [354, 54] is a type II transmembrane [227, 229]

glycoprotein [54, 233]. The N-terminus of Sag has a short, non-essential cytoplasmic tail followed by a transmembrane region [227] of 22 amino acid residues, and a large extracellular portion containing six consensus N-linked glycosylation sites [39, 54, 81, 227, 229, 462], at least one of which must be glycosylated for effective Sag presentation at the cell surface [280]. The extracellular region also includes an MHC class II peptide binding motif (CIIPBM) [200]. The C-terminal region of Sag interacts directly with the hypervariable region 4 (HV4) outside the peptide binding groove [147, 261] and the complementarity determining regions 1 and 2 (CDR1 and 2) of the T-cell receptor (TCR) V β chain [2, 30, 39, 349, 466, 478]; the TCR V α chain has minor indirect effects on this interaction [439, 449, 464]. The TCR V β specificity of Sag is determined by the highly variable 30 to 40 amino acid residues at the C-terminus of Sag [30, 39, 55, 349, 478].

Sag is synthesized in the ER as a 45 kDa glycoprotein containing N-asparagine-linked oligomannosyl carbohydrates with a half-life of 1.5 to 2 hr [233, 462]. During transit through the Golgi complex Sag is modified by the addition of complex-type glycans and proteolyzed at three possible positions by the endoprotease furin and also the endosomal protease cathepsin L [97, 296, 336, 462]. Proteolytic processing is essential for Sag presentation at the cell surface where the 18.5 kDa C-terminal proteolytic fragment remains non-covalently associated with the N-terminal proteolytic fragment that is required for superantigen function [238, 461]. Some reports indicate that Sag associates with MHC class II molecules within the ER and Golgi during biosynthesis, analogous to the invariant chain (Ii)

[199, 462]. Others suggest that Ii prevents Sag-MHC class II association in the ER such that Sag traffics directly via the cellular exocytic machinery to the cell surface where it is efficiently bound and presented by MHC class II-peptide complexes [169]. In the absence of MHC class II, Sag is shed in the culture medium and can undergo paracrine transfer to MHC class II+ cells independent of cell-cell contact [96, 363].

Sag-mediated immune modulation is required for milk-borne transmission of MMTV [3, 379] and may enhance the tumorigenicity of transformed cells through the production of cytokines that support tumor growth [436]. T-cell-mediated immune responses to Sag could also break tolerance and augment immune responses against tumors expressing MMTV Sag [390] while deletion of Sag-reactive T cells can modulate the susceptibility or resistance to infectious and autoimmune diseases [167, 240, 255, 277, 419].

1.6 MMTV replication cycle

The MMTV replication cycle involves a number of stages (Figure 1.3).

1.6.1 Adsorption and cell entry

Retroviral adsorption is facilitated by Env-mediated recognition of a cell surface receptor (Figure 1.3, step 1). A cellular receptor for MMTV, designated as MTR-1, was localized to mouse chromosome 16 [192] while a second ubiquitously expressed candidate receptor, MTR, was mapped on mouse chromosome 19 [163]. The 19 kDa MTR is a novel membrane-associated protein

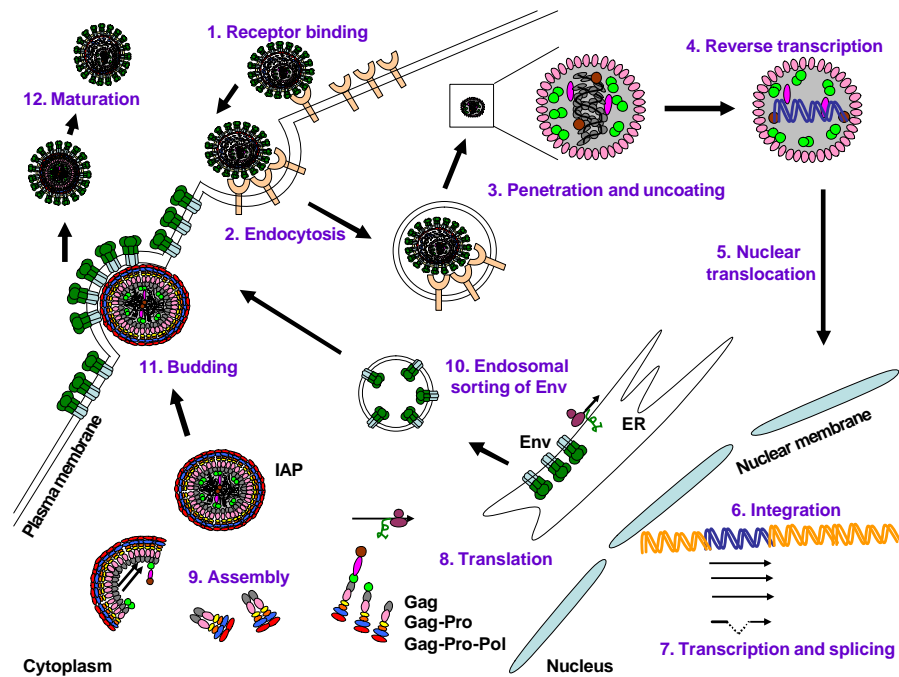


Figure 1.3. MMTV replication cycle.

that contains a hydrophobic domain, a putative N-linked glycosylation site, three O-linked glycosylation sites, and six putative myristoylation sites. Human homologs of the *mtvr* gene have also been described [414]. Most recently, phenotypic screening of the T31 mouse/hamster radiation hybrid panel mapped the transferrin receptor 1 (TfR1) (gene locus on chromosome 16) as the MMTV cell-entry receptor [380]. TfR1 is a type II, single membrane-spanning glycoprotein that is the major means of iron uptake by most cells. Upon ligand binding, TfR1 traffics to the early acidic endosome, where it releases iron, recycles back to the cell surface, and releases transferrin. In addition to transferrin, peptides binding to TfR1 also trigger its endocytosis and traffic into acidic endosomes. Thus, a proposed pathway for MMTV cell entry involves binding to TfR1 at the cell surface followed by

endocytosis via clathrin-coated pits and traffic to acidic endosomes where the low pH would trigger membrane fusion releasing the nucleocapsid into the cytosol (Figure 1.3, steps 2 and 3) [361, 380]. This model is supported by blocks to MMTV infection after treatment of cells with lysomotropic alkalinizing agents [47].

Similar to HIV, following cell entry, the MMTV virion cores might use cytoplasmic dynein and the microtubule network to migrate towards the nucleus and accumulate in the perinuclear region to facilitate access to the host DNA [279].

1.6.2 Reverse transcription

Viral RNA genome is reverse transcribed (Figure 1.3, step 4) by RT to generate a blunt-end, colinear DNA duplex that contains long terminal repeats (LTRs) composed of U3, R, and U5 regions [448]. Two template switches or strand-transfer reactions during reverse transcription generate the LTRs [448]. All the components, except deoxyribonucleotides, required for reverse transcription are present within the virion, and reverse transcription is activated upon entry of the virion core into the cell cytoplasm. Reverse transcription occurs in a ribonucleoprotein complex that includes NC, which enhances RT processivity and template switching [448]. Most retroviruses complete reverse transcription in the cytoplasm; however, some viruses, e.g., avian leukemia virus (ALV), complete reverse transcription following nuclear translocation.

Minus-strand DNA synthesis initiates at the 3'-OH group of a partially unwound tRNA base-paired to the PBS near the 5'-end of the genomic RNA (Figure 1.4, step 1) [448]. DNA synthesis proceeds to the 5'-end of the genomic

RNA to generate the minus-strand strong-stop DNA (-sssDNA) (Figure 1.4). Following RNase H-mediated degradation of the RNA strand in the RNA:-sssDNA duplex (Figure 1.4, step 2), the R sequences at the 5'- and 3'-ends of the viral RNA mediate the first strand transfer of -sssDNA by annealing to the inter- or intramolecular 3'-end of one of the RNAs in the diploid genome (Figure 1.4, step 3). Minus-strand DNA synthesis then continues to the 5'-end of the genomic RNA (Figure 1.4, step 4) accompanied by RNase H digestion of the template RNA, leaving a short polypurine tract (PPT) containing RNA intact (Figure 1.4, step 5). Some viruses may generate additional plus-strand primers from the viral RNA genome.

The PPT RNA primes plus-strand DNA synthesis which halts after a portion of the primer tRNA is reverse transcribed, yielding the plus-strand strong-stop DNA (+sssDNA) (Figure 1.4, step 6). RNase H degrades the primer tRNA exposing the +sssDNA copy of the PBS (Figure 1.4, step 7) that anneals to the complementary PBS on the intramolecular minus-strand DNA completing the second strand transfer (Figure 1.4, step 8). Proviral synthesis is then completed with the plus and minus strands of DNA serving as templates for the complementary strand (Figure 1.4, step 9). The ends of unintegrated viral DNA are defined by the sites where RNase H removes the plus- and minus-strand primers. These cleavages also define the interior boundaries of U3 and U5 in the viral DNA.

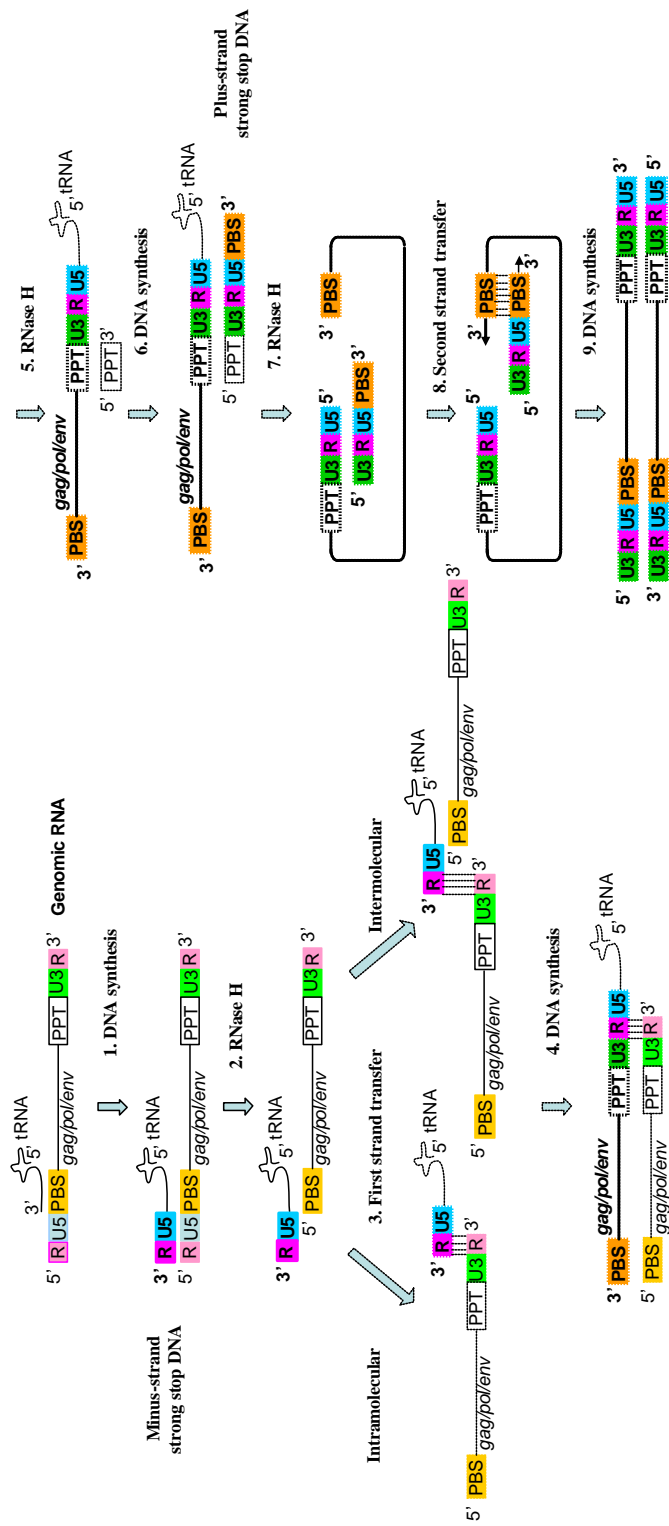


Figure 1.4. Retroviral reverse transcription.

The dimeric nature of the retroviral genome increases the probability of successful reverse transcription while also allowing a high recombination rate [448]. Recombination occurs during reverse transcription according to 2 models - (i) a copy choice model where genetic recombination occurs during intermolecular minus-strand synthesis, and (ii) strand displacement-assimilation model where discontinuous plus-strand synthesis displaces the 5' end of an adjacent nascent plus-strand which can then undergo intermolecular base pairing with the minus-strand DNA and allow recombinant plus-strand synthesis.

1.6.3 Nuclear translocation

Most oncoretroviruses, including MMTV, depend on actively dividing cells for their replication as they gain access to the nucleus during mitosis, when the nuclear membrane is disassembled [448]. The preintegration complex (PIC) is unable to pass through the nuclear pores (Figure 1.3, step 5). HIV, and other lentiviruses, also enter the nucleus during mitosis, but may enter the nucleus during interphase by active transport through the nuclear pore. The viral proteins required for nuclear translocation and integration remain associated with the virions and their replication intermediates.

1.6.4 Proviral integration

A linear DNA copy of the viral genome (provirus) is integrated into host chromosomal DNA (Figure 1.3, step 6) by joining a precise site in the viral DNA to non-homologous sites in the host DNA [265]. The process begins by IN-mediated

cleavage of the 3'-termini of the viral DNA within the viral nucleoprotein PIC. This cleavage eliminates the terminal 2 bases from each 3'-end [109] resulting in recessed 3'-OH groups that provide the proviral attachment sites to the host DNA, and thus define the ends of the integrated provirus. Following 3'-end processing, the PIC translocates to the nucleus [448]. This step precedes the 3'-end processing for viruses that complete reverse transcription in the nucleus. Binding of the host DNA by the viral PIC is followed by an integrase-catalyzed transesterification reaction in which the 3'-OH groups at the viral DNA ends attack the phosphodiester bonds on opposite strands of the target DNA, at positions staggered by 6 bases in the 5' direction for MMTV IN [109, 265]. Gap-filling DNA synthesis by viral RT or host DNA polymerase β or δ extends from the host 3'-OH groups flanking the host-viral DNA junctions [123, 448]. Removal of the 5' dinucleotide overhangs, perhaps by host flap endonuclease I [123], followed by ligation completes proviral integration and duplicates a 6 bp target site sequence flanking the integrated MMTV provirus [109, 265, 448]. The integrated provirus is replicated along with the host-cell DNA, allowing transmission as an integral element of the host genome [448].

Although specific target sequences are not required for integration, large-scale surveys of murine leukemia virus (MuLV) and HIV integration have revealed a strong bias towards transcriptionally active regions of the genome while avian sarcoma-leukosis virus (ASLV) lacks a similar preference for active genes [468]. Highly bent regions of nucleosomal DNA and nuclear matrix-associated chromosomal locations are also strongly preferred as integration sites [448, 468]. The tethering model of integration proposes that host DNA binding proteins interact

with the viral PIC and influence the target site selection [468]. For instance, barrier-to-autointegration factor (BAF) and high mobility group (HMG) chromosomal protein A1 associate with the viral PIC and inhibit autointegration while promoting intermolecular strand transfers [123]. Host DNA binding proteins may also occlude potential target sites [448].

1.6.5 Viral transcription

Retroviral transcription, mediated by the host-cell RNA polymerase II, occurs from the integrated provirus (Figure 1.3, step 7) and is influenced by chromosomal position effects [134, 434, 448]. As previously mentioned (Section 1.5.8), four different MMTV promoters have been described (Figure 1.2) [14, 78, 120, 173, 293, 316, 442, 481]. P_{env2} has elements homologous with promoters of B-cell specific genes like immunoglobulin (Ig) heavy chain, *Ii*, and MHC class II [14]. Expression of *sag* mRNA has been reported from all 4 promoters while all other known MMTV genes and the genomic RNA are mainly transcribed from P_{1196} (the standard LTR promoter).

Standard LTR transcription starts at the U3/R boundary (Figure 1.2) and directs tissue-specific transcription in epithelial cells of lungs, kidney, mammary, salivary and prostate glands and also in Leydig and lymphoid cells [79, 448]. The primary transcripts of the proviral DNA are capped at the 5'-end (methylated GDP attached to the first encoded nucleotide by a 5'-5' linkage), polyadenylated at the 3'-end (50 to 200 noncoding A residues), methylated at several specific sites internally, and in some instances, spliced to generate subgenomic mRNAs [448].

Transcription from P_{1196} terminates at various positions outside the viral template, and 3'-end processing of retroviral RNAs is generated by post-transcriptional endonuclease digestion at the R/U5 border followed by polyadenylation. The sequence AGUAAA in the MMTV U3 region regulates 3'-end processing and polyadenylation [448]. Under most circumstances transcription initiates only from the 5' LTR. This dominance might be due to promoter occlusion as the elongating transcription complexes that originate in the 5' LTR could disrupt or prevent the assembly of transcription factor complexes in the 3' LTR.

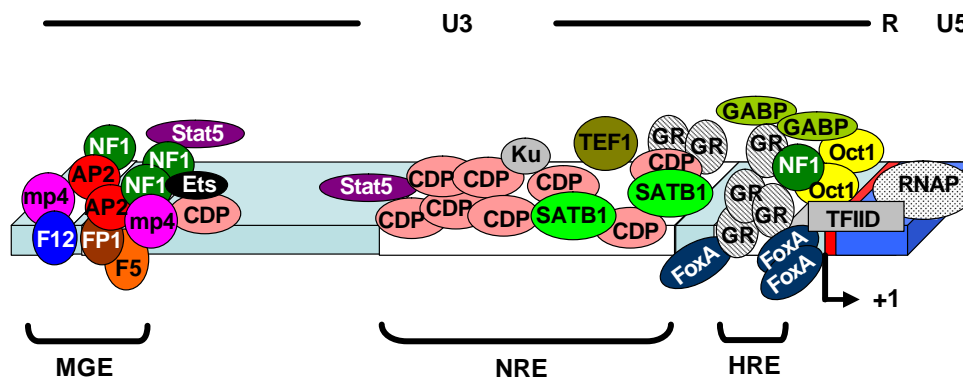


Figure 1.5. Regulatory elements in the MMTV LTR.

The P_{1196} TATA sequence, TATAAAA, is located from -41 to -23 bp upstream of the transcription start site. A basal promoter element located at +2 to +10 from the transcription start site binds the initiation site-binding protein (ISBP) and is required for optimum transcription from the MMTV LTR [344]. Spacing between the TATA box and the ISBP site is important for MMTV promoter function and selection of the transcription start site [132].

Steroid hormones, e.g., glucocorticoids, androgens, and progesterone

enhance MMTV LTR-driven transcription and increase protein production [68, 93, 172, 337, 370, 371, 374, 397, 435, 479]. The hormone responsive element (HRE) located upstream of the transcription start site (-190 to -80) within the LTR (Figure 1.5) provides transcriptional responsiveness to glucocorticoids [204, 267, 343, 387] and also other steroid hormones like androgens, progestins, and mineralocorticoids [69, 70, 180] leading to a dramatic increase in MMTV expression during lactation [299]. The hexanucleotide half-site 5'-TGTTCT-3' and its direct and partial inverted repeats serve as glucocorticoid receptor (GR) binding elements (GREs) [18, 387]. There are 4 GREs within the MMTV HRE, which also bind other hormone receptors that recruit distinct sets of coactivators to the MMTV promoter to upregulate transcription [141, 249, 338].

Hormone induction repositions the 6 nucleosomes (A-F) in a sequence-specific manner on the MMTV LTR and provides differential access to sequence-specific DNA binding proteins, including the octamer transcription factor (Oct-1/OTF-1), nuclear factor 1 (NF1)/CCAAT transcription factor (CTF), and the TATA binding protein (TBP) [9, 10, 87, 223, 368, 428]. Nucleosomes A and B represent the HRE, and binding sites for transcription factors, NF1, Oct-1, and TBP [223]. Oct-1 binds to a region of two 10 bp direct repeats between the positions -60 and -38 immediately upstream of the TATA box (Figure 1.5) and stimulates both basal transcription and hormone induction of transcription from the standard LTR promoter [62, 222, 426]. NF1 binds to the MMTV promoter between -82 to +12 of the transcription start site [87] and is required for glucocorticoid-induced transcription from the MMTV promoter [292].

Hormone induction from the LTR promoter is a bimodal process involving chromatin presetting mediated by weak binding of NF1 and Oct-1 that induces translational re-positioning of the nucleosomes, allowing GR binding and increased basal transcription [183]. Hormone-activated GR binding then triggers a more distinct translational positioning of nucleosomes by recruiting chromatin remodelling and histone modifying complexes that allow a tighter binding of NF1 and Oct1 and transcriptional induction [34, 106]. Strong NF1 binding stabilizes an open nucleosomal conformation to allow efficient binding of more receptor molecules [106]. Prolonged glucocorticoid exposure dephosphorylates histone H1 and inactivates the MMTV promoter [244].

Two regions fp1 (position -139 to -146 from the transcription start site) and fp2 (position -157 to -164 from the transcription start site) show B-cell-specific binding to the heterodimeric Ets factor, GA-binding protein (GABP), which synergizes with the steroid receptor mediated transcriptional upregulation of the MMTV LTR [19]. Ear3/COUP, an orphan member of the steroid/thyroid hormone receptor superfamily of transcription factors, transactivates RNA synthesis by binding to the +102 to +135 region downstream of the transcription start site in the MMTV LTR [215].

The MMTV LTR contains a negative regulatory element (NRE) (Figure 1.5) composed of 3 regions – distal (d) NRE (-645 to -471), junctional (j) NRE (-471 to -365), and proximal (p) NRE (-365 to -264) – which controls tissue-specific expression of MMTV [52, 199, 242, 294, 483]. The transcriptional repressors, special AT-rich-sequence binding protein 1 (SATB1) and the homeodomain protein

CCAAT displacement protein (CDP), bind to the MMTV NRE (Figure 1.5) and repress MMTV transcription in lymphoid cells and the undifferentiated mammary gland, respectively [251, 482, 483, 484]. SATB1 is most abundant in thymus, but it also is expressed in a number of tissues that are semipermissive or nonpermissive for MMTV expression [483]. CDP is expressed in most undifferentiated tissues, yet during differentiation, CDP expression is greatly diminished, thus relieving the repression [483]. The transcription factor Ku recruits DNA-dependent protein kinase in a sequence specific manner to the MMTV NRE (Figure 1.5) resulting in repression of glucocorticoid-induced MMTV transcription [152]. The transcriptional enhancer factor 1 (TEF-1) binds to the pNRE (Figure 1.5) and downmodulates basal and glucocorticoid dependent transcription from the MMTV LTR [263]. MMTV transcription is also inhibited by transforming growth factor-beta ($TGF\beta$), which may be an upstream effector of CDP [71, 291]. The pNRE harbors two GREs [141, 338]

The MMTV LTR harbors a bipartite mammary gland-specific enhancer (MGE) (Figure 1.5) comprised of the BanII element (located between -1075 to -978 or -1078 to -1052 upstream of the transcription start site) and the mammary-specific enhancer of MMTV (MEM) element (-938 to -862 upstream of the transcription start site) [170, 245, 474]. The MGE increases basal and hormone-induced transcription from the MMTV LTR and directs high level expression in the mammary glands of transgenic mice [299, 474]. The BanII element interacts with a multifactor complex containing the mammary gland specific nuclear factor mp4, mp5/AP-2, F3/NF1, and F12 [245, 282]. The MEM element has binding sites

for mammary specific factors, mp4 and Fp1 complex, and ubiquitous factors like signal transducer and activator of transcription 5 (Stat5) or mammary gland factor (MGF), which is activated in response to prolactin [351]. The MEM element also binds mammary cell activating factor (MAF) or Ets, NF-1, AP-2 and F5 complex [171, 351, 458]. The MGE contains prolactin responsive elements, interacts with a factor that binds to the core sequence ACAAAG [181, 295], and overlaps the transcription enhancer for B-cell/myeloid progenitors [366]. Both enhancers share specific functional regions that include the NFI and MAF binding sites [366].

Other *cis*-acting transcriptional elements in MMTV include a 13 bp tannic acid response element in the LTR located downstream of the transcription start site that binds S μ bp-2, a DNA helicase domain containing protein, which negatively regulates transcription from the MMTV LTR [433]. Forkhead transcription factor family member Forkhead box A (FoxA) binds to the MMTV LTR at the positions -232/-221, -57/-46 and -45/-34 [197] and mediates chromatin changes that allow increased basal transcription while decreasing GR-induced transcription.

MMTV also contains transcription regulatory elements outside the LTR. The MMTV *env* gene contains a 411 bp (nt 6801 to 7212) activation-dependent, helper T-lymphocyte-specific transcriptional activator termed MMTV *env* transcriptional activator (META) [293].

1.6.6 Viral translation

Viral proteins are translated from genomic as well as singly and doubly-spliced subgenomic RNAs on free and membrane-bound ribosomes (Figure 1.3,

step 8). Two -1 ribosomal frameshift events are required for complete translation of the genomic MMTV RNA leading to the expression of MMTV Gag-Pro and Gag-Pro-Pol precursors. The dUTPase/Pro coding sequence begins about 16 nt upstream of the Gag termination codon in the -1 reading frame, while the Pol reading frame begins 13 nt upstream of the Pro termination codon [209]. Ribosomal frameshifting occurs when ribosomes traverse a 7-nt sequence termed the shift site [72]. The shift site sequence, host tRNAs, and G-C-rich RNA pseudoknots located 6 to 8 bases downstream of the shift site are critical for determining the efficiency of frameshifting [72, 209]. The *gag-pro* junction specified by the NC stop codon is the first frameshift site where 23% of the ribosomes shift their reading frame to generate the Gag-Pro polypeptide, while 8% of these ribosomes undergo the second frameshift at the *pro-pol* junction to generate the Gag-Pro-Pol polypeptide [209, 300]. The lower frequency of ribosomal frameshifting at the *pro-pol* junction provides a mechanism to regulate the concentration of *gag*-encoded structural proteins and *pol*-encoded enzymes. The three polypeptides Pr77^{Gag}, Pr110^{Gag-Pro}, and Pr160^{Gag-Pro-Pol} are synthesized in the approximate ratio of 30:10:1.

1.6.7 Virion assembly and structure

The MMTV polypeptides Pr77^{Gag}, Pr110^{Gag-Pro}, and a smaller proportion of Pr160^{Gag-Pro-Pol} encapsidate the dimeric viral genomic RNA into a coiled, rope-like structure [302]. This ribonucleoprotein complex forms a spherical immature nucleocapsid core termed intracytoplasmic A particle (IAP) measuring 80 nm in diameter (Figure 1.3, step 9 and Figure 1.6) [283, 303, 480]. Packaging

of the dimeric RNA genome may occur by the RNA switch mechanism wherein NC-binding ψ or E (for encapsidation) sites are sequestered by base-pairing in the monomeric RNA and become exposed following RNA dimerization [110, 448]. Identity of the ψ sequence in the MMTV genome is unknown.

The polyproteins in the IAPs are stabilized by disulfide bonds and are arranged radially with their N-termini near the surface and the C-termini located in the interior [409]. IAPs assemble in virus factories near the nucleus and the plasma membrane [149]. Envelopment occurs by budding through regions of the plasma membrane or the ER that have been modified by the insertion of viral Env proteins (Figure 1.3, steps 10 and 11) with the exclusion of some normal cellular membrane proteins [149, 406, 480]. Gag molecules in the IAPs interact with the TM in the viral membranes via the hydrophobic MA domain [275]. Virion assembly is proposed to be both concentration-driven (based on Gag-Gag interactions) and template-driven (based on envelope/MA and the NC/RNA interactions) [35].

MMTV assembly and budding process interacts with the ubiquitination system as determined from the presence of monoubiquitinated p3-p8 and diubiquitinated NC in MMTV virions [329]. During or shortly after particle budding, the Gag polyprotein is cleaved by the viral protease into mature structural proteins [149, 409]. Cleavage of Gag results in a dramatic structural rearrangement, termed maturation (Figure 1.3, step 12 and Figure 1.6), which is necessary for infectivity. The mature virions also contain cellular constituents, e.g., ribosomes, the primer tRNA associated with the PBS of the viral RNA, and low levels of cellular mRNAs and 5 S ribosomal RNA [149, 340, 448]. Virions also contain

some DNA that might be the early products of reverse transcription [448].

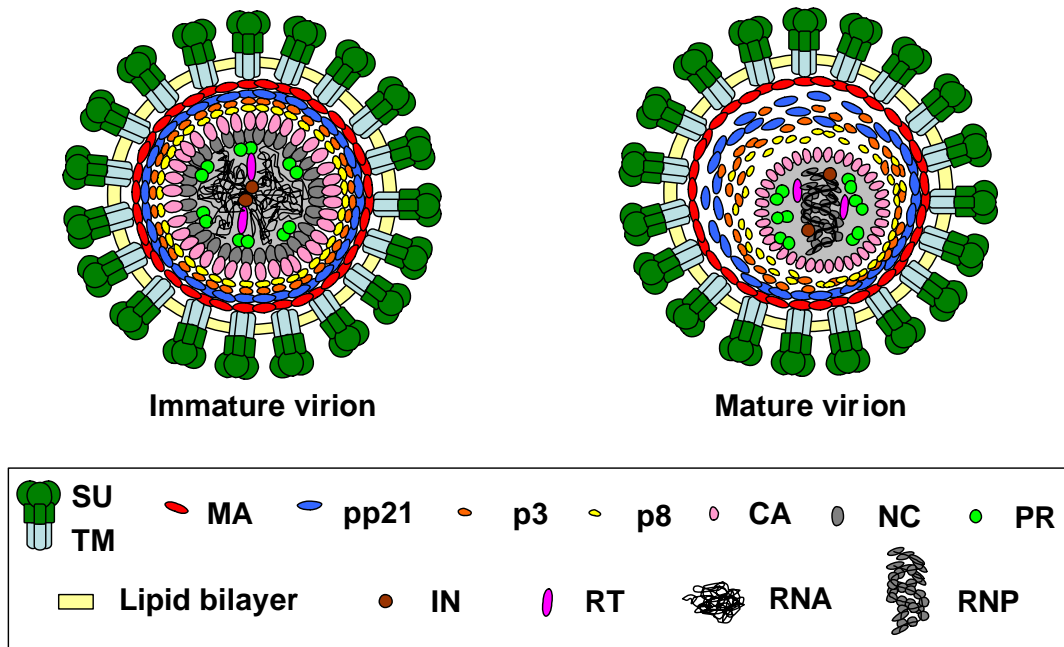


Figure 1.6. MMTV virion structure.

1.6.8 Virion release

Fully assembled MMTV IAPs bud from both the plasma and endosomal membranes (Figure 1.3) and preferentially exit at sites of cell-to-cell contact [150, 303]. Viral infection often induces cell-to-cell contact sites termed virological or infectious synapses that resemble immunological synapses by their high concentrations of adhesion molecules and polarization of the microtubule organization center toward the synapse [303]. These synapses form, at least in part, because Env proteins on the infected cell surface crosslink with their receptors on the target cells [303]. Particles budding at cell-to-cell contact sites are often

elongated and undergo simultaneous endocytosis into the adjacent cell [150, 303]. Retroviral release can also occur directionally in polarized cells [303].

Recent studies have revealed that retroviruses bud by Gag late (L) domain-mediated recruitment of cellular factors involved in the multivesicular bodies (MVB) sorting pathway [303]. Candidate L domains in MMTV Gag include a PSAP sequence in CA and two YXXL motifs in MA and pp21 [329]. Ubiquitination commonly serves as the signal for sorting proteins into MVB vesicles [303]. Membrane distortion during virus budding is achieved through the combined interactions of viral and cellular proteins and, possibly, also specialized lipids [303]. Ubiquitination also plays a role in the release of enveloped viruses. Although Gag proteins of several retroviruses including MMTV are ubiquitinated, its precise role in viral release is not known [303].

1.7 MMTV Transmission

MMTV is an oncogenic retrovirus that causes mammary carcinomas in mice after a long latency (6 to 9 months) [1, 379]. MMTV is transmitted exogenously through milk of infected female mice to the progeny during the first 2 weeks of life [302], or may be inherited in the germline as stably integrated endogenous proviruses (*Mtvs*) [231].

1.7.1 Endogenous MMTVs (*Mtvs*)

Most laboratory mouse strains harbor between 2 to 8 endogenous MMTV proviruses (*Mtvs*) in their germline that follow the Mendelian rules of inheritance

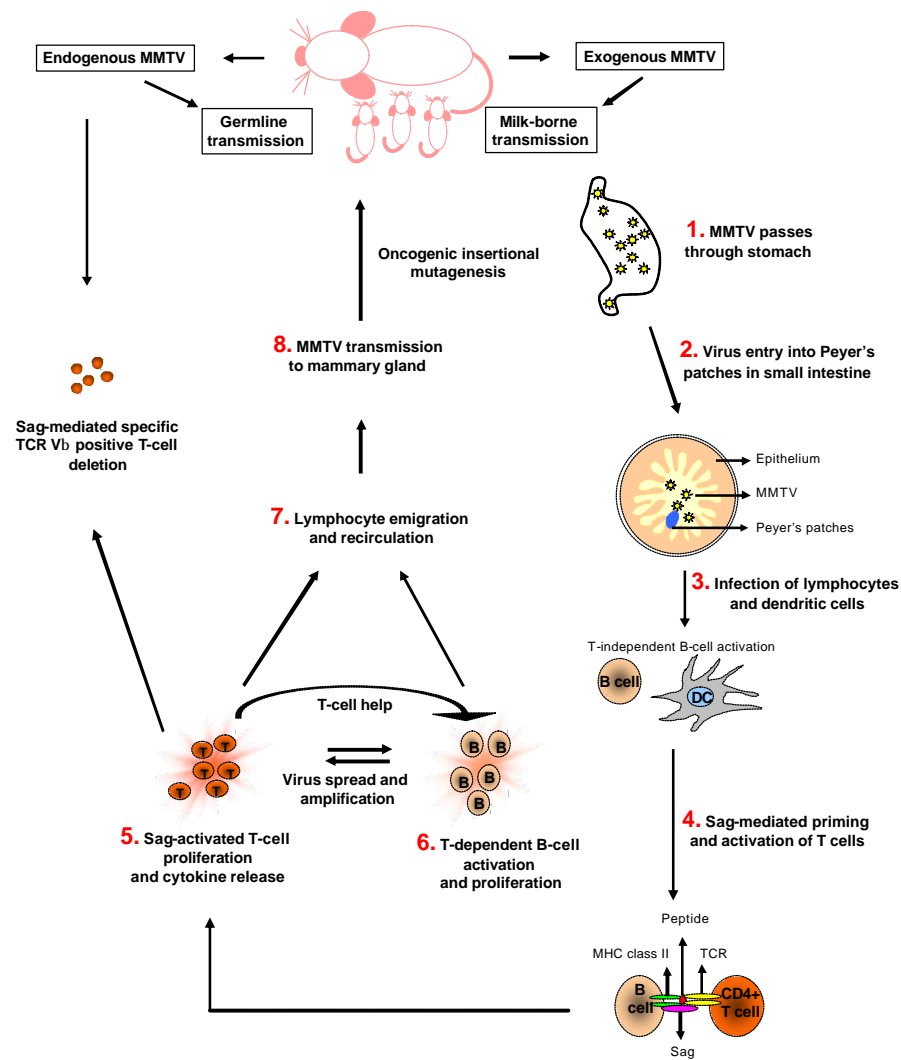


Figure 1.7. MMTV life cycle.

[231]. A majority of the endogenous proviruses are defective for replication and mammary tumorigenesis due to mutations [231]. However, most of these proviruses encode functional Sag that induces intrathymic or peripheral deletion of Sag-reactive T cells altering the mouse T-cell repertoire [31, 388]. Proteins encoded by endogenous *Mtvs* may also serve as self-antigens to generate partial

tolerance against MMTV [4]. Thus, the presence of endogenous *Mtvs* might potentially aid infection by exogenous MMTV. Conversely, it has been proposed that Sag-expressing endogenous *Mtvs* have been maintained in most mouse strains for protection against infection by virulent exogenous MMTVs encoding Sags with similar T-cell reactivities [157, 158, 184].

1.7.2 Exogenous MMTVs

The life cycle of exogenous MMTV is intricately linked with the immune system [4]. While some milk-borne MMTV particles are digested in the stomach [42], other virions enter the Peyer's patches in the small intestine (Figure 1.7, steps 1 and 2) through M cells in the overlying follicle-associated epithelium [176, 218]. Short gastric veins emptying in the superior hilus of the spleen may also serve as portals of MMTV entry [381]. Initial infection is restricted to the Peyer's patches where viral DNA is detectable within 4 days of birth [218]. This infection (until 17 days post-partum) is independent of Sag function and depends on viral reverse transcription and the developmentally regulated proliferation of target cells [186, 345] which primarily include small, resting naive B cells and immature dendritic cells (DCs) (Figure 1.7, step 3) [40, 184, 185, 440]. MMTV can also activate DCs and B cells via the TLR4 [64, 136] and trigger the production of the immunosuppressive cytokine IL10 from B cells, which is stimulated by DCs and macrophages in a TLR4-dependent manner [213]. This immune suppression is critical for the maintenance of infection since in the absence of a functional TLR4, wild type MMTV is eliminated by a cytotoxic T-lymphocyte (CTL) response

directed against Gag epitopes [213]. MMTV infection also results in rapid CD40-CD40L interaction followed by downregulation of both molecules within 24 hr of infection, allowing a balance between virus propagation and host defenses [405].

Antigen presenting cells (APCs) express the MMTV-encoded Sag at their cell surface in association with MHC class II molecules [1, 4, 41] and initiate a helper T-cell response by priming Sag-reactive CD4⁺ T lymphocytes (Figure 1.7, step 4) [1, 4, 11, 201, 281, 348]. DCs are important Sag presenters in the thymic and splenic environments [135] and play an essential role during T-cell priming [29, 256]. Activated B cells upregulate surface MHC class II expression and also present passively acquired Sag [298]. While conventional peptide antigens are recognized on average by 1 in 10⁵ T cells, 5 to 35% of the total mouse T-cell repertoire may be activated by MMTV Sag to undergo rapid proliferation and/or release cytokines (Figure 1.7, step 5) [4, 12]. Sag-mediated stimulation of T-helper cells is essential for virus propagation since most inbred mouse strains are potentially tolerant to MMTV proteins encoded by endogenous *Mtvs* in their genome [256].

Helper-T cells initiate extra-follicular and follicular activation of virus-infected B cells that proliferate vigorously and differentiate into antibody-producing plasma cells (Figure 1.7, step 6) [13, 137, 185, 256]. The antibody response is unable to clear the virus in susceptible mice. MMTV infection also results in IFN α and β production, which limits the early infectious phases and results in longer latency of mammary tumorigenesis. Higher levels of interferon may inhibit the progression of pre-neoplastic lesions [33]. The Sag-mediated immune response results in viral amplification (Figure 1.7) in the absence of reverse

transcription [186] and generates a reservoir of infected and susceptible B and T cells. These lymphoid cells spread the infection from the Peyer's patches to the mesenteric lymph nodes and later to the peripheral lymphoid organs, gut mucosa and epithelial cells of the thymus and the exocrine glands, including the mammary glands in a Sag-dependent manner (Figure 1.7, steps 7 and 8) [137, 138, 164, 184, 218, 274, 318, 345]. Sag also attracts lymphocytes into the draining lymph nodes to enhance the efficiency of viral amplification and spread [278].

Sag activity and MMTV-infected lymphocytes are also required for virus spread within the mammary gland [164]. Disruption of T-B cellular interactions in T- or B-cell knockout mice (Sag transgenic mice [157, 158], nude mice [431], μ MT^{-/-} [40]) or mice lacking co-stimulatory molecules (CD40L^{-/-} [76], CTLA4Ig transgenic mice [73], CD28^{-/-} [331]) reduces or abolishes the Sag-mediated immune response as well as viral spread and transmission. In later stages of chronic MMTV infection, peripheral Sag-mediated T-cell response is reduced since most of the Sag-reactive T cells are gradually deleted following activation (Figure 1.7) [1, 31, 91, 118, 216]. Anergy is maintained in the residual Sag-reactive T-cell population by Sag-specific natural regulatory CD4⁺ CD25⁺ T cells that escape deletion [262, 334]. CD4⁺ T cell-Sag interaction also disrupts B- and CD8⁺ T-lymphocyte homeostasis [356].

1.8 Mechanism of tumorigenesis

MMTV is a non-acutely transforming retrovirus that induces tumors by the stochastic process of insertional mutagenesis. Proviral insertion in the vicinity of a cellular oncogene disrupts the normal organization of the locus and also introduces strong promoter and enhancer elements into the locus that could alter the oncogene expression by several mechanisms [448]. (i) Promoter insertion occurs when a provirus integrates upstream of an oncogene in the same transcriptional orientation. The promoter and enhancer elements in the proviral LTRs may direct increased or developmentally inappropriate levels of oncogene expression [448]. (ii) In enhancer insertion, proviruses inserted upstream of target genes in the opposite transcriptional orientation or downstream from target genes in either orientation can increase gene expression by placing the gene under the influence of strong enhancers within the retroviral LTR [448]. The BanII and MEM elements of the MMTV MGE mediate transcriptional activation of provirus-proximal oncogenes [170]. (iii) During read-through transcription, proviruses integrated within genes may initiate transcripts at the LTR promoter that extend into cellular sequences. Translation of such hybrid transcripts can lead to the expression of altered proteins [448]. (iv) Inactivation of cellular regulatory elements may occur after proviral insertions. Cellular gene expression may be altered by destroying cellular regulatory elements like silencers, pause sites, or polyadenylation signals or lead to altered methylation patterns at the locus [448]. Proviral insertional mutagenesis could also result in the inactivation of tumor suppressor genes [448].

Members of the *wnt* (*wingless*-related MMTV *integration* site), *fgf* (fibrob-

last growth factor), and *notch* gene families among others [66, 326] are common integration sites (CISs) of exogenous MMTV proviruses [86, 128, 305] (Table 1.1) in mammary tumors. The *wnt* genes are a family of 19 or more genes related to the *Drosophila* segmented polarity gene, *wingless* (*wg*) [66]. The *wnt* genes encode cysteine-rich secreted glycoproteins that bind to cell surface receptors. Receptor binding initiates signaling cascades implicated in patterning during embryogenesis and stem cell self-renewal for maintenance of mature tissues [206]. The *fgf* gene family includes 22 members that encode secreted proteins involved in various biological processes, including angiogenesis, mitogenesis, cellular differentiation, cell migration and tissue-injury repair [207]. The four members of the *notch* gene family encode cell surface receptors, which when activated by membrane-bound ligands, influence developmental cell-fate choices in a variety of cellular lineages [214]. Several other potential oncogenes have been identified by directing their expression from the MMTV LTR in transgenic mice (Table 1.2).

The choice of oncogene target and the efficiency of tumor induction is influenced by multiple factors [342] like oncogene cooperativity [448] (Tables 1.1 and 1.2), mouse genetic background [270] and hormonal status [84].

MMTV-encoded proteins may also have a direct role in the tumorigenesis process as suggested by the oncogenic activity of Sag in certain mammary epithelial cell lines [312] and the influence of the *gag* gene on the efficiency of mammary tumor induction by different strains of MMTV [198]. MMTV Env recently was reported to have a transforming activity dependent on the immunoreceptor tyrosine-based activation motif (ITAM) located in SU [219].

Table 1.1. Common integration sites of MMTV in mammary tumors.

Gene	Chromosome	Mechanism of insertional mutagenesis	References
<i>fgf10</i>	13	Enhancer insertion	[424]
<i>wnt1/int1</i>	15	Enhancer insertion	[61, 324, 325, 443]
<i>fgf3/int2</i> ^c	7	Enhancer insertion	[102, 103, 104, 324]
<i>wnt3</i>	11	Enhancer insertion	[376]
<i>notch4/int3</i>	17	Promoter insertion producing a truncated mRNA encoding the Notch intracellular domain	[144, 145, 373, 438]
<i>notch1</i> ^a	2	Transcript truncation generating a 5' <i>notch1</i> transcript encoding the Notch1 ectodomain and promoter insertion producing a truncated mRNA encoding the Notch intracellular domain	[107, 154]
Mitogen activated protein kinase kinase kinase 8 (<i>map3k8/tp12/cot</i>)	18	Enhancer insertion	[126]
<i>wnt10b</i> ^b	15	Enhancer insertion	[182, 243]
<i>hst/fgf4</i> and <i>fgf8</i> ^d	7 and 19	Enhancer insertion	[217, 260, 404]
<i>int5/aromatase</i> (CYP19)	9	Enhancer insertion	[115, 306, 422]
Translation initiation factor eIF3-p48 (<i>int6</i>)	15	Transcript truncation	[17, 271, 297]
Centromere proximal region	11		[357]
Mammary tumor integration site 41 (<i>int41</i>)	6	Promoter insertion	[146]

^a The *notch1* gene was identified as an MMTV common integration site (CIS) in mammary tumors and lymphomas from MMTV-infected MMTV/*neu* and MMTV/*c-myc* transgenic mice [107, 154].

^b The *wnt10b* gene was identified as an MMTV CIS in mammary tumors from MMTV-infected MMTV/*int2* (*fgf3*) transgenic mice [243].

^c The *fgf3* gene was also identified as an MMTV CIS in mammary tumors from MMTV-infected MMTV/*wnt1* transgenic mice [404].

^d The *fgf4* and *fgf8* genes were identified as MMTV CISs in mammary tumors from MMTV-infected MMTV/*wnt1* transgenic mice [217, 260, 404].

Table 1.2. Tumorigenicity of transgenes expressed from MMTV LTR.

Transgenes	Tumor type	References
MMTV/ <i>vegf</i> × MMTV/PyMTAg ^a	Accelerated mammary tumors	[393]
MMTV/ <i>criptol</i>	Mammary tumors	[415]
MMTV/ <i>birc</i>	Ovarian and uterine tumors	[234]
<i>cav</i> ^{-/-} × MMTV/PyMTAg	Accelerated mammary tumors	[460]
MMTV/activated <i>akt1</i> × MMTV/ <i>erb2</i>	Mammary tumors	[202]
MMTV/ <i>csf1</i> × MMTV/ <i>csf1r</i>	Mammary tumors	[224]
MMTV/ <i>muc1</i>	Mammary tumors	[394]
MMTV/MT1-MMP	Mammary tumors	[175]
MMTV/ <i>tgfa</i>	Mammary tumors	[179]
MMTV/PyMTAg	Mammary tumors and lung metastasis	[174]
MMTV/ <i>myc</i>	Mammary, testicular, lymphocytic and mast cell tumors	[413]
MMTV/ <i>myc</i> × p53-null	T-cell tumors	[121]
MMTV/ <i>hras1</i>	Harderian lacrimal gland hyperplasia and focal malignancies of mammary, salivary, and lymphoid tissues	[408]
MMTV/ <i>ccnd1</i>	Mammary tumors	[451]
MMTV/ <i>wnt1</i> × <i>pten</i> -null	Mammary tumors	[248]

Continued on next page

Table 1.2 – continued from previous page

Transgenes	Tumor type	References
MMTV/ <i>rel</i>	Mammary tumors	[377]
MMTV/ <i>hras1</i> × <i>cdknl1a/cip1</i> -null	Accelerated mammary tumorigenesis	[5]
MMTV/ <i>nrg1</i>	Mammary tumors	[232]
MMTV/ <i>egfr</i> (human)	Mammary hyper- and neoplasia	[56]
MMTV/activated <i>kras</i>	Stochastic mammary tumors	[328]
MMTV/ <i>ptpe</i>	Mammary hyperplasia and sporadic tumors	[122]
MMTV/ <i>cdc25b</i>	Mammary hyperplasia	[259]
MMTV/ <i>fgf8</i>	Mammary and salivary gland neoplasia and ovarian stromal hyperplasia	[92]
MMTV/ <i>mmp7</i>	Premalignant hyperplastic alveolar nodules	[382]
MMTV/ <i>mmp7</i> × MMTV/ <i>neu</i>	Accelerated mammary tumors	[382]
MMTV/ <i>myc</i> × MMTV/ <i>bcl2</i>	Accelerated mammary tumorigenesis	[211]
MMTV/ <i>neu</i> × MMTV/p53-172H	Accelerated mammary tumors	[247]
MMTV/ <i>neu</i> × MMTV/ <i>tgfa</i>	Cooperative mammary tumors	[315]
MMTV/ <i>int5</i>	Mammary hyperplasias	[423]
MMTV/ <i>src</i>	Mammary hyperplasias and tumors	[454]
MMTV/ <i>tgfa</i> × MMTV/ <i>myc</i>	Salivary and mammary gland tumors	[7]

Continued on next page

Table 1.2 – continued from previous page

Transgenes	Tumor type	References
MMTV/ <i>int3</i>	Salivary and mammary tumors	[212]
MMTV/ <i>nras</i>	Tumors of multiple tissues	[269]
MMTV/ <i>int1</i>	Mammary and salivary gland tumors	[432]
MMTV/ <i>erbB2</i>	Adenocarcinomas and B-cell tumors	[417]
MMTV/activated <i>neu</i>	Polyclonal mammary tumors	[313]
MMTV/ <i>int2</i>	Mammary and prostate gland hyperplasia	[314]
MMTV/ <i>tgfa</i>	Mammary tumors	[276]
MMTV/ <i>wnt1</i> × MMTV/ <i>int2</i>	Cooperative mammary tumorigenesis	[236]
MMTV/ <i>hras1</i> × MMTV/ <i>myc</i>	Accelerated tumorigenesis	[408]

^a *vegf*: Vascular endothelial growth factor; PyMTAg: Polyoma middle T antigen; *criptol*: Epidermal growth factor-CFC protein family; *btrc*: transducin repeat containing protein (F box protein); *cav*: Caveolin-1; *akt-I*: serine/threonine protein kinase; *erbB2/neu*: erythroblastic leukemia viral oncogene homolog 2; *csf1*: Colony stimulating factor; *csf1r*: CSF-1 receptor; *muc1*: mucin; MT1-MMP: Membrane-type matrix metalloproteinase-1; *tgfa*: Transforming growth factor alpha; *myc*: Myelocytomatosis oncogene; *hras1*: Harvey rat sarcoma virus oncogene 1; *cend1*: Cyclin-D1; *pten*: Tumor suppressor phosphatase and tensin homolog; *ret*: Reticuloendotheliosis oncogene; *cdk1a/cipl*: Cyclin-dependent kinase inhibitor 1A; *nrg1*: Neuregulin-1; *egfr*: Human epidermal growth factor receptor; *kras*: Kirsten rat sarcoma viral oncogene homolog; *ptpe*: Protein tyrosine phosphatase ϵ ; *cdc25b*: Cell division cycle 25 homolog B; *mmp7*: Matrix metalloproteinase matrilysin; *bcl2*: B-cell leukemia/lymphoma 2; p53-172H: p53 dominant oncogenic mutant; *int5*: Cytochrome P450, family 19, subfamily a, polypeptide 1 (*cyp19a1*); *src*: Rous sarcoma oncogene; *int3*: Notch gene homolog 4; *nras*: Neuroblastoma ras oncogene; *int1/wnt1*: *wingless*-related MMTV integration site 1; *int2*: Fibroblast growth factor 3.

1.9 MMTV-associated non-mammary tumors

MMTV has been associated with the induction of non-mammary tumors, primarily of lymphoid origin. Exogenous MMTV proviruses have been detected in T-cell tumors from mouse strains harboring milk-borne MMTV (GR and DBA/2) as well as in strains that lack demonstrable milk-transmitted virus (BALB/cJ and C57BL/6) [94, 112, 113, 241, 289]. However, T-cell tumors often fail to produce MMTV virions although viral RNA and proteins are detectable [112]. MMTV proviruses cloned from these T-cell tumors [for example, type B leukemogenic virus (TBLV), MA, and DL-8] harbor varying deletions within the U3 region of the LTR [27, 112, 113, 199, 237, 241, 290, 473]. These deletions remove the 3'-end of the *sag* gene as well as the NREs in the MMTV LTR; in some cases part of the HRE is lost [27, 199, 237, 241, 290]. Some lymphoma-associated MMTV proviruses also acquire T-cell specific enhancers that may duplicate or triplicate the HRE in the U3 region [27, 286, 473].

Milk-borne MMTV infection has also been associated with the induction of kidney adenocarcinomas in the BALB/cf/Cd mouse strain (a 70% incidence) [457]. Newly integrated MMTV proviruses cloned from these tumors harbor a unique alteration in their LTR U3 region. Starting at the position -333 relative to the transcription start site, 113 bp of the original MMTV LTR have been replaced with a 90 bp unrelated DNA sequence. The deletion and substitution also result in truncation of the NREs and the *sag* gene. In rare cases, MMTV has been reported to induce Leydig cell testicular tumors [355]. MMTV may induce pituitary gland tumors as suggested by the detection of amplified MMTV proviruses in a

pituitary tumor line [355]. Since MMTV is expressed in a variety of epithelial and lymphoid cell types, transcription levels may determine the frequency of insertional mutagenesis.

SJL mice develop germinal center (GC)-derived B-cell lymphomas also called reticulum cell sarcomas (RCS) with >90% incidence within 12 months of age. Endogenous *Mtv29*-encoded Sag plays a critical role in the growth of these tumors by stimulating cytokine production from CD4+V β 16+ T cells [429, 430].

Other immune responses to MMTV have also been implicated in facilitating polyomavirus-induced tumorigenesis. The presence of *Mtv7* in mice of H-2^k background confers susceptibility towards developing polyomavirus-induced tumors [255].

1.10 Type B leukemogenic virus (TBLV)

1.10.1 History

TBLV is a T-cell tropic variant of MMTV that predominantly induces thymic lymphomas in mice [26]. This virus was originally described as a highly leukemogenic virus (DMBA-LV) isolated from a 7,12-dimethylbenz (α)-anthracene (DMBA)-induced thymic lymphoma in a CFW/D strain mouse [21]. The original DMBA-LV isolates contained a 10:1 mixture of type B and type C retroviral RNA while lacking type B-C recombinant genomes [24, 25]. Nucleic acid hybridization demonstrated a high level of relatedness (90%) between the type B retroviral genome and the MMTV(C3H) genome [24]. DMBA-LV-induced lymphoma cell lines and virions released from these cells contained MMTV-related proteins [95].

High levels of type C retroviral proteins, except Env, were also present in these samples. Complete neutralization of viral leukemogenicity by a monoclonal antibody directed against the MMTV Env glycoprotein established the primary role of the MMTV-related type B retrovirus in the induction of thymic lymphomas [25]. In contrast, antibodies directed against the type C retroviral component of DMBA-LV failed to neutralize viral leukemogenicity [25]. Subsequent analysis of DMBA-LV-induced thymic lymphomas revealed the presence of newly integrated MMTV proviruses, whereas newly integrated type C retroviral proviruses were undetectable [94].

1.10.2 Comparison of MMTV and TBLV

Heteroduplex mapping and nucleotide sequence analysis revealed that the major differences between the TBLV and MMTV genomes lie within their LTRs [27]. TBLV has a deletion of 443 nt and a substitution of 124 nt in the LTR U3 region [27]. This deletion encompasses the NREs [52, 242, 251, 252, 304, 482, 483] while the substitution contains triplicate 62 bp elements comprised of the 18 bp 5' flanking sequence apposed to the 44 bp 3' flanking sequence of the deleted region [27] (Figure 1.8). This triplication functions as a T-cell specific enhancer [286].

The TBLV enhancer contains a novel assembly of transcription factor binding sites, combining sites commonly found in MuLV enhancers (c-Myb, GR and Runx1) and in the HIV-1 enhancer (NF- κ B) [287]. GR- and c-Myb-binding sites are critical for the TBLV enhancer function in CD4⁺ T cells but not in CD4⁺CD8⁺ T cells, the major *in vivo* targets of TBLV oncogenesis.

Two unidentified multi-protein complexes, NFA and NFB, and the transcriptional coactivator Aly are also critical for the TBLV T-cell enhancer function. Three other TBLV enhancer-binding proteins, HMG1, HMG2, and hnRNPA1/A2, were identified by mass spectrometry [287]. The role of these proteins on enhancer activity is yet to be characterized.

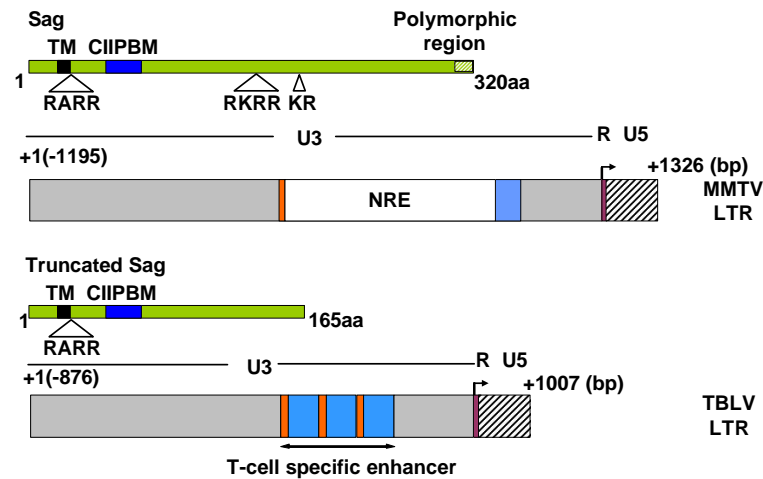


Figure 1.8. Comparison of MMTV and TBLV LTRs.

MMTV and TBLV LTRs (1326 bp and 1007 bp, respectively) are divided into U3, R, and U5 regions. The transcriptional start sites at the U3/R border (1195 bp and 876 bp downstream from the beginning of MMTV and TBLV LTRs, respectively) are denoted by the arrows. The NRE in the U3 region of the MMTV LTR is shown as a white box. The 5' 18 bp and the 3' 44 bp NRE flanking sequences that constitute the triplicate T-cell enhancer in the TBLV LTR are depicted by red and blue boxes, respectively. The Sag protein (320 amino acids) encoded within the U3 region of the MMTV LTR is shown as a green bar above the LTR. The C-terminal hatched box represents the polymorphic 30 amino acid residues that determine the specificity of TCR $V\beta$ chain recognition. The transmembrane (TM) region and the CIIPBM are shown as black and blue boxes, respectively. The three proteolytic processing sites are denoted by triangles below the Sag protein. The NRE deletion and T-cell enhancer substitution in the TBLV LTR truncate the *sag* gene to encode only the first 165 amino acids of MMTV Sag.

1.10.3 TBLV Sag

The LTR deletion and substitution also truncate the *sag* gene [27] encompassing the U3 region of the TBLV LTR (Figure 1.8). The truncated TBLV Sag lacks the entire C-terminal region required for interaction with the TCR [478]. However, TBLV Sag retains the transmembrane region and a portion of the extracellular domain, including the glycosylation sites and a single proteolytic processing site [97, 280]. TBLV Sag retains the CIIPBM and the potential to interact with MHC class II molecules

1.11 Retroviral restriction factors

A number of cellular genes have been identified that actively inhibit retrovirus replication in their mammalian hosts. Among these are the dominant Friend virus susceptibility genes, *Fv1* and *Fv4*, which provide resistance against Friend MuLV [43]. *Fv4* encodes an endogenous retroviral Env protein that blocks infection by receptor interference [43]. *Fv1* encodes a protein that is related to the capsid-like domain of the MuERV-L family of murine endogenous retroviral Gag proteins. *Fv1* restricts viral infection at a post-entry step, most likely by interacting with the incoming viral capsid protein and restricting the viral PIC in the cytoplasm [155]. Many human cell lines express a similar factor termed Ref1 (restriction factor 1) [43, 155] while several primates restrict HIV-1 infection by encoding a related factor termed Lv1 (lentivirus restriction factor 1) [155]. *Lv1* encodes TRIM5 α which belongs to a family of TRIM proteins named for a cluster of tripartite sequence motifs: a RING domain (E3 ubiquitin ligase activity), either

one or two B boxes (zinc binding sequences), and a coiled-coil domain (likely involved in homo- and heterodimerization of the TRIM proteins) [155]. TRIM5 α could potentially target the CA protein of the incoming virus for ubiquitination and degradation. TRIM5 α is also involved in the action of Ref1 and possibly Fv1.

Several other retroviral restriction mechanisms also have been reported. A restriction system that targets retroviruses at an early post-entry step involves enzymes of the APOBEC (apolipoprotein B mRNA editing catalytic subunit) family of cytidine deaminases, in particular APOBEC3G, which remove the amino group from cytosine to form uracil [155]. APOBEC3G is highly specific for single stranded DNA in which C to U conversions result in G to A mutations. The proviral DNA presumably is destabilized due to the subsequent action of uracil N-glycosidases and nucleases such as apurinic/apyrimidinic endonuclease 1 (APE1) [155]. A novel retrovirus resistance gene termed ZAP (zinc finger *antiviral* protein) acts at a late stage of viral infection by blocking the accumulation of viral RNAs in the cytoplasm. ZAP binds viral RNAs and presumably targets them to the RNA exosome, a multiprotein complex involved in cellular mRNA turnover [155].

1.11.1 Modes of MMTV resistance

Several mechanisms of resistance to MMTV infection and tumorigenesis in inbred strains of mice have been described. Increased resistance against mammary tumorigenesis with advancing age of the host and the mammary gland was reported in 1966 [116]. In the same year Riley *et al.* described a phenomenon of interference in which infection of a susceptible mouse strain with the lactate

dehydrogenase elevating virus (LDV) significantly decreased the incidence of spontaneous mammary carcinomas [369]. Reciprocal interference between MMTV and MuLV has also been reported [412]. MuLV infection of milk-borne MMTV-infected BALB/cJ mice resulted in delayed and reduced incidence of MuLV and MMTV-induced tumorigenesis. Interferon production in response to viral infection was proposed to play a major role in this reciprocal interference [32].

An unknown recessive resistance gene in the II-TES mouse strain ensures a low mammary tumor incidence despite the production of high titer, infectious, milk-borne MMTV [8]. Milk-borne transmission of MMTV in the X/Gf mouse strain induces mammary carcinomas with declining incidence until the 7th generation [156]. The cessation of mammary tumorigenesis may be mediated by high levels of antibodies produced against MMTV. Serum antibody response against MMTV has been correlated with resistance to MMTV infection and tumorigenesis in three other instances. In the SWISS and an *Mtv3*⁺ BALB/cHeA congenic mouse strains, the non-tumorigenic expression of the endogenous provirus *Mtv3* protects, at least partially, against exogenous MMTV infection and tumorigenesis by inducing the production of MMTV neutralizing antibodies [177, 178]. Sustained production of virus-neutralizing IgG2a antibodies also provides resistance to MMTV-induced tumorigenesis in I/LnJ mice despite Sag-mediated viral amplification and mammary gland infection [165]. Infected mice also shed antibody coated milk-borne virions that fail to transmit infection to the subsequent generation [350]. The resistance phenomenon is a recessive trait dependent on the production of IFN γ in response to viral infection [350].

Inefficient Sag-cognate T-cell interaction also may produce resistance to MMTV tumorigenesis [347]. The C57BL/6 strain lacks MHC class II I-E molecules that are required for efficient cell surface presentation of MMTV(C3H) Sag [407]. Thus, C57BL/6 mice demonstrate reduced and delayed kinetics of Sag-mediated T-cell deletion and virus production in the mammary gland, resulting in relatively increased resistance to MMTV(C3H)-induced tumorigenesis compared to MHC class II I-E+ strains [465].

Strong Sag-mediated immune responses could also result in protection of mice against mammary tumorigenesis. BALB/c mice infected as neonates with MMTV(SW) develop a high incidence (65%) of mammary adenocarcinomas within 12 months [335]. However, adult mice infected with MMTV(SW) demonstrate reduced mammary gland infection and only a 13% tumor incidence with an average latency of 23 to 24 months. Sag response is inefficient in neonatal mice, and tolerance induction by clonal deletion is delayed. In contrast, adult mice demonstrate dramatic local activation and rapid clonal deletion. Thus, although T-B cellular interaction is required for viral amplification and completion of the life cycle, a strong local immune response to MMTV(SW) in adult-infected mice limits mammary gland infection and protects the mice against mammary tumor development.

A strong immune response also results in resistance against the wild-type MMTV(C3H) and selection of an immune escape variant, MMTV(HeJ), in C3H/HeJ mice [213]. Maintenance of the wild-type virus is dependent on TLR4 signaling, which triggers the production of the immunosuppressive cytokine IL10.

The C3H/HeJ substrain of mice bearing a dominant negative TLR4 mutation eliminates MMTV(C3H) by a cytotoxic immune response directed against Gag [213]. The MMTV(HeJ) recombinant also does not efficiently induce tumors in the C3H/HeJ strain, although it is highly tumorigenic in BALB/cJ mice [198].

Lastly, deletion of cognate T cells by Sags expressed from endogenous *Mtv* loci can protect mice against infection by tumorigenic exogenous MMTV strains encoding Sags with similar T-cell reactivities [157, 184]. However, endogenous *Mtvs* might potentially aid infection of mice by exogenous MMTV strains by altering the T-cell repertoire [388] and generating a partial tolerance against exogenous MMTV proteins [4].

1.12 Comparison of *Vibrio cholerae* and MMTV pathogenesises

V. cholerae is a motile, Gram-negative, curved rod belonging to the family *Vibrionaceae* [362]. These bacteria are natural inhabitants of aquatic ecosystems and are facultative human pathogens. Infection usually starts by ingestion of food or water contaminated with *V. cholerae*. Subsequently the bacteria must pass through and survive the gastric acid barrier of the stomach, then penetrate the mucus lining that coats the intestinal epithelia [362]. The surviving bacteria adhere to and colonize primarily the small intestinal epithelial cells, eventually producing cholera toxin (CT) and causing diarrhea.

Normal adult mammals (other than humans) are not colonized by *V. cholerae*, yet these bacteria colonize the small intestines of several suckling mammals, including mice, presumably due to immature host immune defenses

[226]. The majority of cholera symptoms in humans are caused by CT. *V. cholerae* strains lacking CT genes have a markedly higher 50% lethal dose (LD₅₀) in suckling mice, but display no colonization defects. *V. cholerae* strains that produce CT induce a highly secretory diarrhea that develops in the absence of inflammation, while strains that do not produce CT cause milder diarrhea, but the disease is more inflammatory in nature [386].

CT belongs to the AB₅ family of toxins [246]. Five identical 11-kDa peptides associate in a ring-like structure to form the pentameric B-subunit (55 kDa). The B-subunit binds to the oligosaccharide domain of a membrane glycolipid receptor, ganglioside GM1, at the cell surface resulting in the association of CT with lipid rafts. The single A-subunit assembles non-covalently with the B-subunit and is cleaved into A1- and A2-chains, which remain linked by a disulfide bond and extensive non-covalent interactions. The toxin is internalized by clathrin-dependent and -independent mechanisms in different cell types. CT is then transported from the plasma membrane to the ER by a lipid-sorting pathway. In the ER lumen, the A1 subunit is activated by thiol-dependent reduction followed by unfolding and disassembly from the rest of the toxin. The A1 subunit is transported to the cytosol where its ADP-ribosylating activity targets the host cell G-protein Gs α . ADP-ribosylated Gs α constitutively activates adenylate cyclase activity, leading to increased levels of intracellular cyclic adenosine monophosphate (cAMP), which inhibits active sodium chloride absorption and increases chloride and bicarbonate secretion. The result is passive water loss, leading to a marked decrease in intravascular volume, hypotension and hypoperfusion of critical organs and, in

severe cases, death ensues with a high mortality rate (>20%). Interestingly, cAMP is also reported to upregulate glucocorticoid-induced expression of MMTV [476].

Like MMTV Sag, CT acts as an immunomodulator that suppresses host innate immunity and skews the development of an adaptive immune response. However, CT inhibits the production of tumor necrosis factor α (TNF α) and nitric oxide (NO) by macrophages, thus inhibiting innate immunity at the earliest steps of infection. TNF α is a proinflammatory cytokine that induces endothelial cells to express chemokines and cell adhesion molecules to recruit neutrophils and other leukocytes to the site of infection. CT also downregulates the production of IL12 by macrophages and reduces T-cell responsiveness to IL12 by inhibiting the expression of surface receptor chains IL12R β 1 and IL12R β 2. Through suppression of the major Th1-associated cytokine, CT inhibits the amplification of a cell-mediated immune response. On the other hand, CT stimulates DC maturation for priming of a Th2 immune response. The primary role of CT in a *V. cholerae* infection may be modulation of host immune responses.

1.13 Rationale for these studies

MMTV is an oncogenic retrovirus that causes mammary tumors in mice after a long latency (6 to 9 months) [3, 379]. In contrast, TBLV causes thymic T-cell lymphomas with a short latency (42 to 60 days) when introduced intrathymically into neonatal mice [26]. TBLV is a unique virus for study despite sharing a very high degree of sequence similarity with MMTV, because of its altered tissue and disease tropism. The cellular oncogene targets of TBLV and MMTV also appear to

be different. A common integration site of TBLV, termed *Tblvi1*, was mapped to a 53 kb locus on the mouse X chromosome in 20% of primary TBLV-induced tumors [311]. The *c-myc* and *Rorc* genes are common integration sites for TBLV in thymic T-cell lymphomas, while MMTV frequently targets members of *wnt*, *fgf*, and *notch* gene families among others [60, 66, 358]. Therefore, the factors that control disease and tumorigenic specificity are key to understanding retroviral pathogenesis.

In vivo studies have been previously performed by intrathymic injection of neonatal mice with heterogeneous TBLV-containing tissue culture supernatant from a T-cell lymphoma line, 485-10 [21, 22, 23, 375]. T-cell lymphomas were observed in these mice at an incidence of approximately 90% [26]. To further understand the biology of this virus, a tumorigenesis assay in adult mice was developed in our laboratory by using a cloned TBLV hybrid provirus (HYB-TBLV) created by replacing a portion of the 3' LTR of an MMTV clone with the corresponding region from the TBLV LTR (section 3.1). Lymphomagenicity of HYB-TBLV demonstrated that the determinants of TBLV lymphotropism lie within the LTR. Lymphomagenicity of two other T-cell tropic MMTV variants, MA and DL-8, have been previously studied in adult mice in the context of molecular viral clones [475]. Similar to TBLV, these variant LTRs also harbor NRE deletions and T-cell enhancers that truncate the *sag* gene [286, 473]. Thus, mutant MMTV and TBLV hybrid proviruses were used to assess the contributions of the truncated TBLV Sag, NRE deletion and T-cell enhancer acquisition towards the conversion of mammotropic MMTV to a lymphomagenic virus (section 3.1).

Endogenous *Mtvs* integrated in the germline of most inbred mouse strains

are known to prevent infection by exogenous MMTV strains bearing Sags of similar T-cell specificities. The endogenous *Mtvs* can recombine with exogenous MMTV strains and influence the infection cycle of exogenous MMTVs with disparate Sag specificities. Therefore, we were motivated to develop a well characterized inbred mouse strain (termed BALB/*Mtv*-null) that lacks endogenous *Mtvs* capable of recombination with engineered exogenous MMTV strains under investigation. Surprisingly, the endogenous *Mtv*-free BALB/*Mtv*-null mice were resistant to MMTV-induced mammary tumorigenesis and TBLV-induced lymphomagenesis. These mice also were resistant to the cholera toxin-producing strain of *Vibrio cholerae*. This novel mouse strain was characterized to identify novel pathogen restriction pathways. Description of such pathways could elucidate new aspects of the pathogen replication cycle as well as host-pathogen interactions. Knowledge of restriction factors would also allow the identification of potential therapeutic targets in the pathogen replication cycle.

Materials and Methods

2.1 Mice

BALB/cJ [H-2^d, *Mtv*6, 8, 9] and PERA/Ei (H-2^k [446], endogenous *Mtv*-free [446]) mice were obtained from Jackson Laboratories (Bar Harbor, ME) while exogenous MMTV(C3H)+ C3H/HeN mice were obtained from Harlan (Indianapolis, IN). BALB/*Mtv*-null (H-2^d) mice lacking endogenous *Mtvs* were selected from a BALB/cJ x PERA/Ei cross followed by 10 backcrosses to the BALB/cJ strain. These mice have been maintained for numerous generations by standard brother-sister mating. The BALB/*Mtv*-null strain was backcrossed to the BALB/cJ strain to derive BALB/cJ x BALB/*Mtv*-null F1 mice heterozygous for *Mtv*6, 8, and 9 endogenous proviral loci (Figure 3.19). BALB/cJ congenic strains that were homo- or heterozygous for a single endogenous *Mtv* locus (BALB/*Mtv*6, BALB/*Mtv*8, and BALB/*Mtv*9 mice collectively termed BALB/*Mtv*-SP) were derived by backcrossing the BALB/cJ x BALB/*Mtv*-null F1 (NE1) strain to the BALB/*Mtv*-null strain followed by inbreeding of selected NE2 strains (Figure 3.19). Siblings of BALB/*Mtv*-SP mice from the NE3 generation that lacked endogenous *Mtvs* were designated as BALB/*Mtv*-null_{SP} to indicate the similarity

of their genetic derivation with the BALB/*Mtv*-SP mice (Figure 3.19). All mouse strains were bred and maintained in the Animal Resources Center at the University of Texas at Austin. Sentinel mice were tested periodically to ensure the absence of common bacterial and viral pathogens. Some mice were bled under anesthesia at 1 to 3 month intervals from their retro-orbital sinus using heparinized Natelson blood collecting tubes (Fisher Scientific, Pittsburgh, PA). Mouse breeding, maintenance, PCR typing, inoculations and bleeding were performed by Mary Lozano.

2.2 Mouse injections and foster nursing

Weanling (4 to 5 week-old) mice were intraperitoneally injected with a single dose of 2×10^7 rat XC fibroblast cells or Jurkat human T cells stably expressing wild-type or mutant MMTV/TBLV hybrid proviruses using a 23 gauge needle. For some experiments weanling mice were inoculated intraperitoneally with 5×10^6 A20 B cells or A20 cells stably transfected with the infectious provirus, pHYB-MTV [403] (A20/HYB-MTV). Prior to inoculation into mice, the cells were washed twice with phosphate-buffered saline [PBS] (137 mM NaCl, 3 mM KCl, 8 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2 mM KH_2PO_4 , pH 7.4) and resuspended in 500 μl of PBS. Aliquots of the cells inoculated into mice were analyzed by Western blotting to verify the expression of MMTV proteins. XC cells stably transfected with pHYB-MTV (XC/HYB-MTV) were induced for 24 hr before injection into mice by supplementing the culture medium with 10^{-6} M dexamethasone (Sigma Chemical Laboratories, St. Louis, MO).

BALB/cJ and BALB/*Mtv*-null pups were foster-nursed on exogenous

MMTV(C3H)+ C3H/HeN females by substituting them for some age-matched C3H/HeN pups within 24 hr of birth. All injected and foster-nursed females were bred regularly to stimulate lactogenic hormones.

2.3 Cell lines

Jurkat human T-cell leukemia line (clone E6-1) [456] was obtained from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in complete medium containing RPMI medium (Gibco®, Invitrogen Corporation, Carlsbad, CA) supplemented with 7.5% (v/v) heat-inactivated (30 min at 56°C) fetal bovine serum (FBS) (Gibco®, Invitrogen Corporation), 2 mM L-glutamine (Gibco®, Invitrogen Corporation), 50 µg/ml gentamicin sulfate (Abbott Laboratories, North Chicago, IL), 100 µg/ml streptomycin (Gibco®, Invitrogen Corporation), and 100 U/ml penicillin (Gibco®, Invitrogen Corporation). The HC11 normal murine mammary epithelial cell line derived from BALB/c mice [28] were obtained from Dr. Jeff Rosen (Baylor College of Medicine, Houston, TX). This cell line was maintained in complete medium containing RPMI supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin sulfate, 0.5 µg/ml insulin, and 0.5 µg/ml epidermal growth factor (Invitrogen Corporation). XC/HYB-MTV cells are a rat fibroblast cell line [418] stably transfected with the hybrid proviral clone of MMTV, pHYB-MTV [403]. The derivation of these stable transfectants has been described previously [316]. Briefly, XC cells were co-transfected with 5 µg of CsCl-purified pYB-MTV and 0.05 µg of the plasmid, pTR174, expressing the

hygromycin resistance cassette, using 10 μ l of DMRIE-C (Invitrogen Corporation). Transfections were performed in triplicate using six-well plates, and the cells were selected in Dulbecco's modified Eagle's medium (DMEM) (Gibco[®], Invitrogen Corporation) containing 7.5% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 500 μ g/ml hygromycin B (Invitrogen Corporation) until discrete colonies were established. The colonies in the three wells were pooled, expanded, and maintained in DMEM substituted with 5% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml gentamicin sulfate, 2 mM L-glutamine, and 250 μ g/ml hygromycin B (Invitrogen Corporation). XC/HYB-MTV cells were treated with 10^{-6} M dexamethasone for 24 hr to induce MMTV production prior to inoculation into mice. The BALB/c derived B-cell lymphoma line, A20 [221], (obtained from Dr. Bridget Huber, Tufts University, Boston, MA) was maintained in RPMI medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin 100 μ g/ml streptomycin, 50 μ g/ml gentamicin sulfate, 2 mM L-glutamine and 50 μ M β -mercaptoethanol. All cell lines were grown in a humidified incubator maintained at 37°C and 7.5% CO₂.

2.4 Plasmid preparation

2.4.1 Large-scale plasmid preparation

Large-scale plasmid preparations were performed by alkaline lysis and CsCl/ethidium bromide equilibrium centrifugation as described by Sambrook *et al.* [383]. Briefly, 500 ml of Luria-Bertani (LB) broth (1% tryptone w/v, 0.5% yeast extract w/v, 0.5% NaCl w/v, pH 7.4) was inoculated with 20 ml of an overnight

culture of the bacterial transformant grown in LB broth supplemented with the appropriate antibiotic. The culture was incubated at 37°C with aeration by shaking at 250 rpm to density equivalent to an A_{600} (absorbance at 600 nm) of 1.2. Freshly prepared chloramphenicol solution in 100% ethanol was added to the culture to a final concentration of 170 $\mu\text{g/ml}$ followed by overnight incubation at 37°C with aeration (shaking at 250 rpm). Bacteria were collected by centrifugation at 6000 rpm for 10 min at 4°C using the JA-10 rotor in the Avanti®-JE centrifuge (Beckman Coulter Inc., Fullerton, CA). The bacterial pellet was thoroughly resuspended in 7 ml solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) followed by transfer to a 50 ml conical tube. Bacterial lysis was performed by adding 1 ml lysozyme solution to a final concentration of 2 mg/ml followed by a 10 min incubation at room temperature. The solution was mixed with 16 ml of freshly prepared solution II (0.2 N NaOH, 1% w/v SDS) and incubated at room temperature for 10 min. The lysate was neutralized with 12 ml cold solution III (3 M potassium acetate solution) followed by thorough mixing and a 10 min incubation at 4°C. The suspension was centrifuged at 3800 rpm for 30 min at 4°C in the 243 swinging bucket rotor of the IEC Centra CL3R centrifuge (International Equipment Company, Needham Heights, MA). The supernatant was transferred to a 50 ml conical tube, and nucleic acids were precipitated after a 20 min incubation with 0.6 volume of isopropanol at room temperature. Precipitated nucleic acids were recovered by centrifugation at 3800 rpm for 30 min at 25°C in the IEC Centra CL3R centrifuge. The pellet was washed once with 10 ml of 70% ethanol, air-dried for 5 min, and resuspended in 3.5 ml of TE 10:10 (10 mM Tris-HCl, pH 7.4 and

10 mM EDTA, pH 8.0). CsCl (4.6 g) was dissolved into this suspension followed by the addition of 680 μ l of ethidium bromide solution [5 mg/ml]. The suspension was centrifuged at 3800 rpm for 15 min at 4°C in the IEC Centra CL3R centrifuge. The supernatant was transferred to an OptiSeal™ ultracentrifuge tube (Beckman Coulter Inc.) and centrifuged at 50,000 rpm for 18 hr at 20°C in the NVT 65.2 near vertical tube rotor using the L7 centrifuge (Beckman Coulter Inc.). The plasmid DNA band was transferred to a 15 ml conical tube using a 5 ml syringe and an 18 gauge needle. The ethidium bromide was extracted 3 to 4 times with 2 volumes of G50 buffer [10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 2 mM EDTA] saturated n-butanol. Two volumes of TE 10:10 were added, and the solution was adjusted to 0.2 M NaCl prior to addition of 2 volumes of cold 100% ethanol. After 15 min incubation at 4°C, the DNA was recovered by centrifugation at 2850 g for 30 min at 4°C in the IEC Centra CL3R centrifuge. Following a 70% ethanol wash and 5 min of air-drying, the pellet was resuspended in 2 ml of TE 10:10. Contaminating RNA was removed by incubation with 100 μ g/ml pancreatic RNase A (Sigma) and 200 units of RNase T1 (Sigma) at 37°C for 30 min. The solution was incubated with 100 μ g/ml of proteinase K (Sigma) in the presence of 0.5% SDS for 30 min at 37°C. Following phenol:chloroform extraction, the solution was dialyzed using a 10,000 molecular weight cut-off (MWCO) Slide-A-Lyzer® dialysis cassette (Pierce, Rockford, IL) against TE 10:1 with 3 buffer changes. Plasmid DNA was recovered by ethanol precipitation and resuspended in TE 10:0.1 following a 70% ethanol wash.

2.4.2 Small-scale plasmid preparation

Small-scale plasmid preparation was performed according to the alkaline lysis protocol described by Sambrook *et al.* [383]. Bacterial cultures were grown overnight at 37°C by shaking at 250 rpm in 2 ml LB broth supplemented with the appropriate antibiotic. Bacteria were collected from 1.5 ml of the culture transferred into a 1.8 ml Eppendorf tube by centrifugation at 13,000 rpm for 2 min at room temperature in the Sorvall® Biofuge pico centrifuge (Kendro Laboratory Products, Asheville, NC). The bacterial pellet was resuspended in 250 μ l of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing 100 μ g/ml pancreatic RNase A followed by lysis with 250 μ l of freshly prepared solution II (0.2 N NaOH, 1% SDS) and neutralization with 350 μ l of cold solution III (3 M potassium acetate solution). The lysate was centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant was transferred to a 1.8 ml Eppendorf tube and 600 μ l of isopropanol was added to precipitate the nucleic acids, which were then recovered by centrifugation at 13,000 rpm for 15 min at room temperature. The pellet was washed with 70% ethanol, air-dried for 5 min and resuspended in 50 μ l of TE 10:0.1.

2.5 Plasmids

pHYB-TBLV. The TBLV molecular clone, pHYB-TBLV, (made by Dr. Jaquelin Dudley) was constructed by substituting the *Cla*I to *Sst*I region of the 3' LTR of pHYB-MTV with the corresponding region from the TBLV LTR. The gel purified *Cla*I to *Sst*I fragment of the plasmid pTBLV-LUC [286] containing

the TBLV enhancer triplication was ligated to a gel-purified fragment of pHYB-MTV [403] that had been digested completely with *Sst*I and partially with *Cla*I. After transformation of DH5 α cells, the 3' LTR of the MMTV provirus containing the TBLV enhancer region was recovered in the plasmid backbone of pHYB-MTV (pTBLV 3'PRO). pTBLV 3'PRO was digested to completion with *Cla*I and treated with alkaline phosphatase. The phosphatase-treated fragment was ligated to a gel-purified 9.2 kb fragment containing the majority of the pHYB-MTV provirus obtained after complete digestion of pHYB-MTV with *Sst*I and partial digestion with *Cla*I. Following transformation of DH5 α and screening, a full-length proviral clone (pHYB-TBLV) was obtained.

The hygromycin resistance cassette within the 1.5 kb *Xho*I fragment from pUHD15-1-Hygro (-) (kindly provided by Dr. Paul Bates) was introduced at the *Nhe*I site of both pHYB-TBLV and pHYB-MTV (by Dr. Farah Mustafa).

pHYB-TBLV_{sagDFS}. This plasmid (made by Dr. Farah Mustafa) was prepared by introducing frameshift mutations at the *Avr*II and *Cla*I restriction enzyme sites in the 3' LTR of pHYB-TBLV. The *Cla*I site within the TBLV LTR in the plasmid pTBLV-LUC was digested, and the ends were repaired using Klenow DNA polymerase. The resulting linearized plasmid was re-ligated and digested with *Avr*II and *Sst*I. The 0.6 kb fragment obtained from this digestion was ligated to the 16 kb fragment of pHYB-TBLV digested with *Avr*II and *Sst*I. The resulting clone was then digested with *Avr*II, the overhangs were repaired using Klenow DNA polymerase, and the linearized plasmid was re-ligated to generate the plasmid pHYB-TBLV_{sagDFS}.

pTBLV_{sag}DFS-LUC. The 3' LTR of pHYB-TBLV_{sag}DFS was amplified by PCR using KlenTaq polymerase (AB Peptides, St. Louis, MO) with the primers (Table 2.1) TBLVENV8509(+) containing a *Bgl*II restriction site and MMTVLTR1302(-) containing an *Nhe*I restriction site. The product was gel purified using the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) and digested with *Bgl*II and *Nhe*I followed by repair of the overhangs using Klenow DNA polymerase. This PCR product was ligated to the promoterless firefly luciferase (LUC) expression construct, p19LUC [444], which had been digested with *Hind*III and treated with Klenow DNA polymerase followed by removal of the 5' phosphate groups with alkaline phosphatase.

pMTVA_fE-LUC and pMTV_{Stu}E-LUC. The TBLV T-cell enhancer was amplified by PCR using KlenTaq polymerase from pTBLV-LUC with the primers (Table 2.1) TBLVENH_{Alu}(+) and TBLVENH_{Alu}(-). The product was gel purified using the QIAquick gel extraction kit and digested with *Alu*I followed by gel purification. The pMTVA_fE-LUC plasmid was obtained by ligating this PCR product with the gel purified pMTV-LUC plasmid (previously described as pC3H-LUC [52, 286]) that had been digested with *A*fII followed by subsequent treatments with Klenow DNA polymerase and alkaline phosphatase. The product also was ligated with the gel purified pMTV-LUC plasmid that had been digested with *Stu*I and treated with alkaline phosphatase to generate pMTV_{Stu}E-LUC.

pMTV Δ NRE-LUC. The construction of this plasmid (previously termed pd6) has been described [286].

pRL-TK. This plasmid (obtained from Promega, Madison, WI) contains the

herpes simplex virus (HSV) thymidine kinase (TK) promoter upstream of the sea pansy (*Renilla reniformis*) luciferase gene.

pHYB-MTV Δ fE, pHYB-MTV Stu E, and pHYB-MTV Δ NRE. The *Avr*II to *Sst*I fragments of the corresponding LTRs were obtained from pMTV Δ fE-LUC, pMTV Stu E-LUC, and pMTV Δ NRE-LUC. These gel purified fragments were individually substituted for the *Avr*II to *Sst*I fragment within the 3' LTR of the pHYB-MTV plasmid containing the hygromycin resistance cassette.

All restriction enzymes and Klenow DNA polymerase were purchased from New England Biolabs (NEB) (Beverly, MA). T4 DNA ligase used for ligation reactions was purchased from Invitrogen Corporation. Calf intestinal alkaline phosphatase was obtained from Roche (Basel, Switzerland) and Promega (Madison, WI). All primers were synthesized by Integrated DNA Technologies (IDT), Coralville, IA. All selected clones were confirmed by sequencing at the Institute for Cellular and Molecular Biology (ICMB) DNA sequencing facility at the University of Texas at Austin.

2.6 Stable transfections

Jurkat/HYB-TBLV and Jurkat/HYB-TBLV sag DFS cell lines (made by Dr. Farah Mustafa [317]) were obtained by transfecting Jurkat T cells with 2 μ g of pHYB-TBLV or pHYB-TBLV sag DFS plasmids (purified by the CsCl method described in Section 2.4) using Superfect (Qiagen Inc.) according to the manufacturer's instructions. The cells were plated in 6-well plates and transfected in triplicate. Stably transfected cells were selected in 0.2 mg/ml hygromycin B

(Invitrogen Corporation) in complete medium five days following transfection. Stably transfected cells arising in the triplicate wells were pooled, expanded and maintained in complete medium supplemented with 0.2 mg/ml hygromycin B.

Jurkat T cells that stably express HYB-MTV, HYB-MTV Δ flE, HYB-MTV Δ StuE, or HYB-MTV Δ NRE (termed Jurkat/HYB-MTV, Jurkat/HYB-MTV Δ flE, Jurkat/HYB-MTV Δ StuE, and Jurkat/HYB-MTV Δ NRE, respectively) were obtained by electroporation of CsCl-purified plasmid preparations. Jurkat cells (split in a 1:3 ratio one day prior to electroporation) were resuspended in complete medium at 2.5×10^7 cells/ml. The cell suspension was aliquoted (400 μ l) into electroporation cuvettes (BTX Instrument Division, Harvard Apparatus Inc., Holliston, MA) with a 4 mm gap size with 30 to 40 μ g of purified plasmid DNA. The cells were incubated with the DNA for 10 min at room temperature and then subjected to electroporation at 1050 μ F, 720 ohms, and 260 V using the BTX Electro Cell Manipulator (ECM) 600 (Harvard Apparatus Inc.). Each plasmid was electroporated in triplicate. Following a 10 min rest at room temperature, the cells were plated in 6 ml of complete medium in 60 mm Petri dishes. At 48 hr post-transfection, cells from the triplicate transfections were pooled and plated in a 100 mm Petri dish in 20 ml complete medium supplemented with 250 μ g/ml hygromycin B and, cultured for three weeks in the presence of hygromycin B. These cells were subsequently maintained in hygromycin B-containing complete medium. A portion of the pooled cell population was used to prepare DNA and analyzed by PCR to confirm the identity of the proviral integrants. Western blotting and reverse transcription (RT)-PCR (followed by sequencing) was used to assess the expression of the viral

proteins and mRNA, respectively.

A20/HYB-MTV stable transfectants were obtained by electroporation of A20 cells with 20 μg of the plasmid pHYB-MTV that contains the MMTV molecular clone [403] and a hygromycin resistance cassette (provided by Dr. Farah Mustafa). Electroporation was performed in triplicate using the BTX ECM 600. Twenty four hours prior to electroporation, A20 cells were plated at a density of 6×10^5 cells/ml. For electroporation, the cells were washed once with RPMI medium, and resuspended to 3.8×10^7 cells/ml in RPMI. DNA purified by CsCl method was added to 400 μl of the cell suspension and placed in a sterile 4 mm cuvette (BTX Instrument Division, Harvard Apparatus, Inc.). The cell suspension was incubated at 25°C for 10 min and then electroporated at 280 V, 975 μF , and 72 ohms. Following electroporation, the cells were incubated on ice for 10 min and then plated in 6 ml complete medium in 60 mm Petri dishes. At 48 hr post-transfection, cells from the triplicate transfections were pooled and plated in 20 ml of fresh complete medium supplemented with 400 $\mu\text{g}/\text{ml}$ hygromycin B (Invitrogen Corporation) in a 100 mm Petri dish for three weeks. The concentration of hygromycin B was determined by the survival of A20 cells in different concentrations of the drug.

2.7 Transient transfections

All plasmids used for transfection were prepared by the CsCl method. Jurkat T cells (split at a 1:3 ratio one day prior to transfection) were transiently transfected in triplicate using Superfect (Qiagen) according to the manufacturer's instructions.

Six-well plates were plated with 2.5×10^6 Jurkat cells/well in 2.5 ml complete medium. The transfection mix/well of the six-well plate was prepared in 75 μ l of RPMI by adding 2 μ g of the LTR-LUC reporter plasmid (pTBLV_{sagDFS}-LUC, pMTV-LUC, pTBLV-LUC, pMTVA_{flE}-LUC, pMTV_{StuE}-LUC, or pMTV Δ NRE-LUC), 0.1 μ g of the co-transfectant pRL-TK, and 10 μ l of Superfect. The transfection mix was incubated at room temperature for 10 min and then applied drop-wise to the well. The six-well plate was swirled to evenly distribute the transfection mix and incubated at 37°C in 7.5% CO₂. The firefly and *Renilla* luciferase activities were measured 48 hr post-transfection.

HC11 cells were transfected twice in triplicate with 10 μ g of the LTR-LUC plasmids [pMTV-LUC, pTBLV-LUC, pMTVA_{flE}-LUC, pMTV_{StuE}-LUC, or pMTV Δ NRE-LUC] and 2 μ g of pRL-TK by electroporation using the BTX ECM 600 electroporator. HC11 cells (split 1:3 one day prior to transfection) were trypsinized, washed once with RPMI, and resuspended in RPMI at 5×10^7 cells/ml. Plasmids were then added to 200 μ l aliquots of cells placed in 2 mm electroporation cuvettes. The DNA was mixed with the cells and incubated at room temperature for 10 min. The cells were then electroporated at 1750 μ F, 140 V, and 72 ohms. Following 10 min at room temperature, the cells were plated in 5 ml of complete medium in 60 mm tissue-culture dishes and incubated at 37°C in 7.5% CO₂. At 24 hr post-transfection, 10^{-6} M dexamethasone (DEX) was added to a triplicate set of transfections, while the other triplicate set was maintained without DEX induction. All cells were harvested at 48 hr post-transfection and assayed for firefly and *Renilla* luciferase activities.

2.8 Reporter gene assay

Firefly and *Renilla* luciferase luminescence was measured with the Dual-Luciferase[®] Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, cells were washed twice in PBS and lysed in 50 μ l of passive lysis buffer by 3 cycles of freezing and thawing. The lysate was clarified by centrifugation at 13,000 rpm for 1 min at room temperature, and the protein concentration was measured by the Bradford method [51] using the Bradford protein assay reagent from Bio-Rad Laboratories (Hercules, CA). Forty micrograms (20 μ l) of the lysates were assayed for the firefly and *Renilla* luciferase activities using the TD-20e Luminometer (Turner Designs, Inc., Sunnyvale, CA). The firefly luciferase activity of each sample was normalized for DNA uptake by dividing by the *Renilla* luciferase activity in the same sample. The normalized firefly luciferase activity in each sample was then re-calculated relative to the normalized firefly luciferase activity from the wild-type MMTV LTR (assigned a value of 1). The mean (\pm standard deviation) derived from the triplicate relative firefly luciferase activities of each plasmid was plotted.

2.9 Preparation of retrovirus containing culture supernatants

Retrovirus-containing culture supernatants were prepared by growing Jurkat/HYB-MTV, Jurkat/HYB-TBLV or Jurkat/HYB-TBLV_{sagDFS} cells at a high density [$1-2 \times 10^7$ cells/ml] for 18 hr in complete medium lacking hygromycin B. The cells were removed by centrifugation at 3800 rpm for 15 min at 4°C in

the IEC Centra CL3R centrifuge. The retrovirus containing supernatant was stored at -85°C in aliquots. In some instances, the retroviral supernatant was further concentrated by centrifugation at 20,000 rpm for 1.5 hr at 4°C in ethanol-sterilized Ultra-Clear centrifuge tubes using the SW 55 Ti rotor in the L7 centrifuge (Beckman Coulter Inc.). The supernatant was discarded, and the pellet was resuspended overnight with shaking at 4°C in complete medium at 1/100th of the original volume of the culture supernatant. Culture supernatants from 485-10 cells were obtained from a laboratory stock.

2.10 SDS-PAGE and Western blotting

Cells were washed twice in PBS followed by lysis in 50 to 100 μ l of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% v/v Triton-X100, 1% v/v sodium deoxycholate, 0.1% SDS) that was supplemented with 50 μ l/ml β -mercaptoethanol (Sigma-Aldrich Corporation), 1 μ l/ml aprotinin (Sigma-Aldrich Corporation), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich Corporation). The cell lysate was pelleted at 13,000 rpm for 10 min at 4°C, and the protein concentration in the supernatant (whole cell lysate) was measured using the Bradford protein assay reagent from Bio-Rad Laboratories [51]. Loading buffer [6X: 0.35 M Tris-HCl, pH 6.8, 10.28% (w/v) SDS, 30% (v/v) glycerol, 0.6 M dithiothreitol, 0.012% (w/v) bromophenol blue] was added at a final concentration of 3X to the whole cell lysate containing 100, 50, or 20 μ g of protein prior to boiling for 2 min. For Western blot analysis of virions in the culture supernatants, 3X loading buffer was used for varying volumes of the culture supernatants and

boiled for 2 min. The proteins were resolved on 8% SDS-PAGE gels according to the protocol described by Sambrook *et al.* [383] and transferred at 150 mA to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) overnight at 4°C in Western transfer buffer (39 mM glycine, 48 mM Tris base, 1% (w/v) SDS, 20% (v/v) methanol). The membrane was blocked with TBST [20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% (v/v) Tween-20] containing 10% (w/v) nonfat milk for 1 hr at room temperature on a rotary shaker. The membrane was then incubated with the primary antibody [MMTV CA-specific mouse monoclonal antibody (MAb) [350] or actin-specific goat antiserum diluted 1:50 or 1:500, respectively, in TBST containing 1% (w/v) nonfat milk] for 1 hr at room temperature on a rotary shaker. The membrane was then subjected to three washes with TBST (10 min each) and incubated with horseradish peroxidase (HRP) conjugated secondary antibody [anti-mouse immunoglobulin G (heavy + light chain), IgG(H+L), or goat immunoglobulin-specific antibody] (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), diluted 1:8000 in TBST containing 1% (w/v) nonfat milk, for 1 hr at room temperature on a rotary shaker. The membrane was washed thrice for 10 min each with TBST, and the antibody bound protein bands were visualized by Western Lightning enhanced chemiluminescent (ECL) reagent (Perkin Elmer, Wellesley, MA) according to the manufacturer's instructions.

The specificity of MMTV-specific mouse antisera was determined by Western blotting against purified MMTV virions [National Institutes of Health (NIH), Bethesda, Maryland]. MMTV virions (750 µg) were boiled in an equal volume of 6X loading buffer, separated on a preparative 8% SDS-PAGE gel, and

the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with TBST containing 10% (w/v) nonfat milk for 1 hr prior to use in a Mini-PROTEAN II multiscreen apparatus (Bio-Rad Laboratories). Mouse serum dilutions [1:20 in 200 μ l of TBST containing 1% (w/v) nonfat milk] were applied to the membrane through the channels and incubated for 2 hr at 25°C on a rotary shaker. As positive controls for the Western analysis, 200 μ l of a 1:50 dilution [in TBST containing 1% (w/v) nonfat milk] of MMTV CA- and SU-specific monoclonal antibodies were also applied to the membrane. The membrane was washed twice with TBST before release from the multiscreen apparatus. Following three more washes with TBST, the membrane was incubated with HRP-conjugated secondary antibody [anti-mouse IgG(H+L)] diluted 1:8000 in TBST containing 1% (w/v) nonfat milk. Following a 1 hr incubation with shaking at room temperature, the membrane was washed thrice with TBST, and the antibody-bound protein bands were visualized using the Western Lightning ECL reagent (Perkin Elmer).

2.11 Flow cytometry

Peripheral blood lymphocyte (PBL) analysis. Mice were bled from their retro-orbital sinus at three-month intervals. PBLs were isolated from 200 μ l of blood that was layered on 500 μ l of Histopaque mixture containing a 1:1.21 ratio of Histopaque 1119-1 and Histopaque 1077-1 (Sigma) followed by centrifugation at 1000 g for 20 min at 25°C. The PBLs were washed with cold fluorescence activated cell sorting (FACS) wash buffer (FWB) containing PBS supplemented with 2.5% (v/v) heat-inactivated FBS and 0.1% (w/v) sodium azide. Cells were collected by

centrifugation at 450 g for 10 min at 4°C, and the pellet was resuspended in 30 μ l of FWB. The cell suspension was distributed into the required number of aliquots in alternate wells of a round bottom 96-well tissue culture plate and incubated in the dark for 30 min on ice with 100 μ l of the antibody solution containing an optimal dilution (determined experimentally) of the MAb in FWB. Following antibody labeling, the cells were washed thrice with 200 μ l of cold FWB, resuspended in 150 μ l FWB, and analyzed on the FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using CELLQuest software. In some instances, the labeled cells were washed thrice with 200 μ l of PBS before fixation in 200 μ l of 1% paraformaldehyde in PBS and stored at 4°C until analysis.

For estimating the proportion of Sag-reactive T cells, the PBLs were dually stained for cell surface markers CD4 and TCR V β 3, -5, -7, -8, -12 or -14 using phycoerythrin (PE) conjugated anti-mouse CD4 MAb (RM4-5) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse TCR V β 3 (KJ25), V β 5.1 and 5.2 (MR9-4), V β 7 (TR310), V β 8.1 and 8.2 (MR5-2), V β 12 (MR11-1) or V β 14 (14-2) MAbs from PharMingen (San Diego, CA). The percentage of TCR V β + cells in the gated CD4+ T-cell population was calculated. Multiple animals were analyzed for each time point. The average percentage (\pm standard deviation) of CD4+TCR V β + T cells was plotted.

Tumor cell population analysis. Single cell suspensions prepared from tumor samples were labeled for cell surface markers CD4, CD8 α and Thy1.2 using PE-conjugated MAb against mouse CD4 (RM4-5), CyChrome-conjugated MAb against mouse CD8 α (53-6.7), and FITC-conjugated MAb against mouse Thy1.2

(53-2.1) from PharMingen. The cells were analyzed on the FACSCalibur Flow Cytometer using CELLQuest software.

2.12 Genomic DNA preparation

Tissue fragments were incubated in 2 to 3 ml and cell lines were incubated in 1 ml of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% w/v SDS, 100 μ g/ml fresh proteinase K) at 55°C for 1 to 2 days. If the lysates were very viscous, additional proteinase K and lysis buffer were added during this incubation. The lysates were then extracted once or twice with phenol/chloroform, and the aqueous phase was collected in a separate tube. DNA was precipitated using ethanol and the genomic DNA was spooled out into an Eppendorf tube using a Pasteur pipette. The DNA was washed with 70% ethanol, resuspended in TE 10:0.1, and stored at 4°C.

2.13 Southern blotting

Southern blotting was performed as described previously [288]. Genomic DNA (15 μ g) was digested overnight with the appropriate restriction enzyme (*Hind*III for clonality analysis of lymphomas and *Pst*I for determining the LTR structure of proviruses in virus-induced tumors) according to the manufacturer's (NEB) recommendation. The digested DNA was subjected to electrophoresis on a 0.8% agarose gel, photographed following ethidium bromide staining, and then subjected to depurination in 0.125 M HCl for 15 min. After rinsing with

water the DNA was subjected to denaturation for 30 min in a solution containing 0.5 M NaOH and 1.5 M NaCl followed by neutralization in a solution of 1.5 M NaCl and 0.5 M Tris-HCl, pH 7.0. The DNA was transferred overnight and subjected to UV crosslinking on a nitrocellulose membrane (HYBOND™-XL) (Amersham Biosciences, Piscataway, NJ). The membrane was incubated at 42°C in 7 ml of hybridization buffer containing 5X SSPE [0.9 M NaCl, 0.05 M NaH₂PO₄, 0.05 M EDTA], 50% v/v formamide, 0.5% w/v SDS, 100 µg/ml salmon sperm DNA, 100 µg/ml yeast RNA and 5X Denhardt's solution [1X: 1 g/l Ficoll, 1 g/l polyvinylpyrrolidone, 1 g/l bovine serum albumin - pentox fraction V]. Radioactively labeled probes were prepared by random priming using hexameric primers extended by Klenow DNA polymerase using a deoxyribonucleotide triphosphate (dNTP) mix containing ³²P-dCTP (Perkin Elmer). Previously described probes [288] p86T5β and Cγ1.2 were used for assessing the clonality of T-cell tumors based on TCRβ and γ chain rearrangements, respectively. The LTR structure of proviruses in virus-induced tumors was determined using labeled probes prepared from the entire MMTV LTR (released from pMTV-LUC by *Hind*III digestion) and the MMTV NRE (the *Stu*I to *Afl*II fragment of the MMTV LTR). The membranes were incubated overnight at 42°C with 4.8 ml of the hybridization buffer containing 10 ng/ml of the probe labeled to a specific activity of ca. 10⁸ cpm/µg. The membranes were washed five to six times with buffer containing 2X to 0.1X SSC (20X SSC: 3 M NaCl, 0.3 M sodium citrate) and 0.1% w/v SDS and subjected to autoradiography.

2.14 Polymerase chain reaction (PCR)

Genomic DNA (1 μ g) isolated from Jurkat/HYB-MTV, Jurkat/HYB-MTV Δ flE, Jurkat-HYB-MTV Δ StuE, and Jurkat/HYB-MTV Δ NRE was subjected to PCR analysis using KlenTaq DNA polymerase according to the manufacturer's instructions. The following primer (Table 2.1) pairs were used for the PCR analyses - LTR403(+) with C3HLTR978(-); C3HLTR961(+) with MMTVLTR1302(-); and C3HLTR727(+) with MMTVLTR1302(-). All primers were synthesized by IDT. The PCR products were gel purified using the QIAquick gel extraction kit and verified by sequencing.

The presence and identity of endogenous *Mtvs* in mice was determined by PCR as described previously [31]. Briefly, genomic DNA was isolated from tail clips of individual mice according to a previously described protocol [31], and endogenous *Mtv* sequences were amplified by priming from the variable portion of the MMTV LTR and/or the DNA sequences flanking the proviruses. Initial PCR assays were performed using PCR Supermix (Gibco BRL, Gaithersburg, MD) and was subsequently replaced with JumpStart REDAccuTaq™ LA DNA polymerase (Sigma-Aldrich Corporation) according to protocols specified by the manufacturer.

2.15 RNA preparation

RNA was prepared according to the guanidine isothiocyanate method described by Chomczynski and Sacchi [83]. Mouse tissue was homogenized in the appropriate volume (100 mg tissue/ml) of TRI reagent [2 M guanidine

Table 2.1. Primers used for cloning, PCR, and RT-PCR analyses.

Primer	Sequence
TBLVENV8509(+)	5' AGCCTTGACCAAGTGCAGTCAGATCTTAACGTG 3'
MMTVLTR1302(-)	5' GACTAGGCTAGCTGCCGCAGTCGGCCGACCTGAGGG 3'
TBLVENHAlu(+)	5' AATAGAAAGAGACGCTCAAGCT 3'
TBLVENHAlu(-)	5' GTCTCACAAGCTTGTTTTAA 3'
LTR403(+)	5' CCAATAGGTAGATTATTAGTTACT 3'
C3HLTR978(-)	5' AGGTTGGGCCAATAAAGGCTATTCA 3'
C3HLTR961(+)	5' CCTTTATTGGCCCAACCTT 3'
C3HLTR727(+)	5' AAAGTGTTATATAGATCCCTCCC 3'
C3HLTR501(-)	5' GAGGTTGAGCGTCTCTTTCTATT 3'
C3HLTR501(+)	5' AATAGAAAGAGACTCTCAACCTC 3'
C3HLTR1074(-)	5' AACACTCAGAGCTCAGATCAGAACC 3'
TBLVLTR786(-)	5' CACTCAGAGCTCAGATCAGAAC 3'
gag79(+)	5' TCATGTGAAAGAGAGTAGTGCAATAGAA 3'
gag461(-)	5' ATCTGCCTTCTCCTTTTCTTCATATGGTTC 3'
C3H230(+)	5' CATCACAAGAGCGGAACGGAC 3'
env796(-)	5' GCTTATCTACTTGAAAGCAGCCT 3'
GAPDH427(+)	5' CATGTTTGTGATGGGTGTGAACCA 3'
GAPDH983(-)	5' GTTGCTGTAGCCGTATTCATTGTC 3'

isothiocyanate, 12.5 mM sodium citrate, pH 7.0, 0.25% sarkosyl, 0.05 M β -mercaptoethanol, 0.2 M sodium acetate, pH 5.2, and 50% phenol, pH 7.5, saturated with diethyl pyrocarbonate (DEPC)-treated water] using a Kinematica Polytron homogenizer (Brinkmann, Westbury, NY). The homogenate was centrifuged at 2850 g for 20 min at 4°C in the IEC Centra CL3R centrifuge, and the supernatant was transferred to a 15 ml conical tube. The phase separation reagent, 1-bromo-3-chloropropane (BCP), (Molecular Research Center, Inc., Cincinnati, OH) was added to a concentration of 100 μ l/ml of homogenate. The solution was vigorously shaken, incubated for 15 min at room temperature, and subjected to centrifugation

for 30 min at 2850 g (4°C). The aqueous phase was transferred to a fresh 15 ml conical tube, and RNA was precipitated by adding isopropanol to a concentration of 0.5 ml/ml of the original volume of TRI reagent. For isolation of RNA from glycogen-rich tissues, like liver, RNA precipitation was performed with a 1:1 mixture of isopropanol and a salt solution (1.2 M NaCl, 0.8 M sodium citrate) added to the aqueous phase at the concentration of 0.5 ml/ml of the original volume of TRI reagent. After 10 min at room temperature, the RNA was collected by centrifugation at 2850 g for 30 min at 4°C. The RNA pellet was washed once in 70% ethanol, air-dried, resuspended in DEPC-treated water and transferred to an Eppendorf tube. DNA and low-molecular-weight RNA were removed by high-salt precipitation of the RNA [332] by adding sodium acetate, pH 5.6, to a final concentration of 3 M. The RNA was collected by centrifugation at 13,000 rpm for 15 min at 4°C. The pellet was washed once with 70% ethanol, air-dried, resuspended in DEPC-treated water and stored at -80°C. For longer periods, the RNA was stored as an ethanol precipitate by adding 0.3 M sodium acetate and 2.5 volumes of cold 100% ethanol.

To extract RNA from cell lines, cells were disrupted in TRI reagent (10^7 cells/ml) by repeated pipetting. The protocol detailed above was used for purification of the RNA from the lysate. All solutions used for RNA preparation and manipulation were treated overnight with 0.1% DEPC and autoclaved.

2.16 Reverse transcription (RT)-PCR

Total RNA (20 μ g) isolated from normal or malignant mouse tissue or cell lines was treated with 3U of amplification grade DNase I (Invitrogen Corporation) at 37°C for 30 min in the presence of an RNase inhibitor. DNase I was inactivated using 2.5 mM EDTA and incubation at 68°C for 20 min. The RNA was incubated with 400 U of Moloney murine leukemia virus (M-MLV) RT (Invitrogen Corporation) and poly-dT₁₇ as primer in 50 μ l as recommended by the manufacturer. Reactions without RT were performed to verify the lack of DNA contamination. The resulting cDNA (5 μ l) or the minus-RT reaction was used in a 50 μ l reaction containing 50 mM Tris-HCl (pH 9.1), 16 mM ammonium sulphate, 3.5 mM MgCl₂, 150 mg/ml BSA, 0.3 mM dNTPs, 100 ng or 20 pMol each of forward and reverse primers, and 2.5 units of KlenTaq. Forty cycles of amplification, including 1 min denaturation at 94°C, annealing at 55°C for 1 min, and extension at 72°C for 1 min, were performed in a PTC-100™ Programmable Thermal Controller (MJ Research Inc., Reno, NV). The following primer (Table 2.1) pairs were used for RT-PCR analyses of Jurkat/HYB-MTV, Jurkat/HYB-MTV Δ fE, Jurkat/HYB-MTV Δ StuE, and Jurkat/HYB-MTV Δ NRE cells and tumor RNA - TBLVENV8509(+) with C3HLTR501(-); C3HLTR501(+) with C3HLTR1074(-); C3HLTR727(+) with C3HLTR1074(-); C3HLTR501(+) with C3HLTR978(-); TBLVENV8509(+) with TBLVLTR786(-); and C3HLTR501(+) with TBLVLTR786(-). RT-PCR analysis of MMTV-infected BALB/*Mtv*-null tissues was performed using the following primer (Table 2.1) pairs: all MMTV cDNAs, TBLVENV8509(+) with C3HLTR501(-)

and C3HLTR501(+) with C3HLTR1074(-); MMTV *gag* cDNA, gag79(+) with gag461(-); MMTV *env* cDNA, C3H230(+) with env796(-). As a control for RNA integrity, RT-PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (*gapd*) mRNA was performed, as above, with 1 μ l of cDNA using primers (Table 2.1) GAPDH427(+) and GAPDH983(-). RT-PCR products were analyzed by electrophoresis on 2% agarose gels. The expected RT-PCR products were purified using QIAquick gel extraction kit and sequenced. In some instances, the gel-purified RT-PCR products were cloned into pGEM[®]-T Easy vector (Promega) according to the manufacturer's instructions and then sequenced. All RT-PCR primers were synthesized by IDT.

For some experiments, restriction fragment length polymorphisms (RFLPs) were used to differentiate the RT-PCR products amplified from exogenous MMTV(C3H) versus endogenous *Mtvs* using the primers TBLVENV8509(+) and C3HLTR501(-) (Table 2.1). RT-PCR products were purified through Micro Bio-Spin P-30 columns (Bio-Rad Laboratories) and digested with *Cla*I (NEB) for 2 hr at 37°C. An internal plasmid control for *Cla*I digestion was included in each reaction. The restriction digests were analyzed by electrophoresis on 2% agarose gels. The expected fragments were gel purified and sequenced.

2.17 Mouse serum preparation

Mice were bled from their retro-orbital sinus and 200 μ l of non-heparinized mouse blood was allowed to clot for 4 hr at room temperature. Samples were incubated overnight at 4°C to allow clot retraction, and the serum was separated

by centrifugation at 2750 g for 10 min at 4°C.

2.18 ELISA: Enzyme-linked immunosorbent assay

MMTV virion proteins were used to detect MMTV-specific antibodies in mice sera using a previously described [350] ELISA protocol with some modifications. All wells of Nunc-Immuno™ 96 Microwell™ Maxisorp™ plates (Nalge Nunc International, Rochester, NY) were coated with 10 µg/ml of purified MMTV virions (National Institutes of Health) in 50 µl of borate buffered saline (125 mM NaCl, 167 mM boric acid, pH 8.0) overnight at 4°C. The coating buffer was then removed, and the wells were washed twice with the wash buffer [0.05% (v/v) Tween-20 in PBS]. The excess wash buffer was removed by tapping the plates and the wells were incubated for 2 hr at 37°C in a humid atmosphere with 150 µl of the blocking buffer [0.05% (v/v) Tween-20 and 1% (w/v) bovine serum albumin (BSA) in PBS]. Two-fold sera dilutions (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640) were prepared in 30 µl of the dilution buffer [0.05% (v/v) Tween-20 and 0.25% (w/v) BSA in PBS] and added in duplicate columns of wells after removal of the blocking buffer. The positive controls consisted of 30 µl each of a 2-fold dilution series (1:60, 1:120, 1:240, 1:480, 1:960, 1:1920, 1:3840) of the MMTV CA-specific monoclonal antibody [350] added to duplicate columns of wells. The last well of each column received 30 µl of the dilution buffer alone. The plates were incubated for 2 hr at room temperature and then washed thrice with the wash buffer. The HRP-conjugated secondary antibody against mouse IgG(H+L) (Jackson ImmunoResearch Laboratories, Inc.) (50 µl at 1:8000 in dilution buffer)

was added to each well and incubated for 1 hr at room temperature. The wells were washed thrice with the wash buffer followed by three washes with PBS. Wells then received 100 μ l of the substrate solution [phosphate/citrate buffer, pH 5.0 (50 mM Na₂HPO₄, 24 mM sodium citrate), 0.01% (w/v) 3,3',5,5'-tetramethylbenzidine dihydrochloride (Sigma-Aldrich Corporation), 6% H₂O₂], and the plates were incubated in the dark at room temperature for 15 min. The enzyme reaction was stopped by addition of 25 μ l of 2 M H₂SO₄ to each well followed by measurement of absorbance at 450 nm. The antibody titer was calculated as the reciprocal of the highest serum dilution that reacted specifically with the MMTV virions.

2.19 *Vibrio cholerae* lethal dose 50 (LD₅₀) assay

V. cholerae strain 0395 colonies were isolated on an L agar [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar] plate supplemented with 67 μ g/ml streptomycin sulfate. A single colony was inoculated into 3 ml of L broth containing 67 μ g/ml streptomycin sulfate and cultured overnight at 37°C with shaking at 250 rpm. Subcultures (diluted 1:100) were then established in 3 ml L broth without the antibiotic and allowed to grow to an A₆₅₀ of 0.5. This culture was estimated to contain approximately 5×10^8 colony forming units (CFU)/ml of bacteria. A 1:10 dilution of the culture in PBS was used to prepare 1 ml bacterial suspensions in PBS supplemented with 0.02% (w/v) Evan's blue dye containing 2×10^6 and 2×10^7 CFU/ml of bacteria. A suspension containing 2×10^8 CFU/ml of bacteria was prepared by pelleting the bacteria from the required volume of the original undiluted culture at 13,000 rpm for 2 min. The

pellet was resuspended in PBS containing 0.02% (w/v) Evan's blue dye. Each bacterial suspension was then distributed into 1.8 ml Eppendorf tubes in 50 μ l aliquots. BALB/cJ, BALB/*Mtv*-null and BALB/*Mtv*-SP pups (5 to 6 days old) were intragastrically inoculated with 50 μ l of the bacterial suspension (dose = 10^5 , 10^6 or 10^7 CFU) using a 1 ml syringe and a 23 gauge needle covered with a 1 inch long, sterile intramedic polyethylene tubing after smoothing of the open end. The pups were observed for 48 hr for death and morbidity. Serial dilutions were prepared from the 3 bacterial suspensions used for mouse inoculations, and the appropriate dilutions (100 μ l) were plated on L agar plates containing 67 μ g/ml streptomycin sulphate. The CFU/ml of bacteria was then calculated according to the formula:

$$CFU/ml = \frac{\text{Number of colonies} \times \text{Dilution factor} \times 1000\mu l}{100\mu l} \quad (2.1)$$

The LD₅₀ of *V. cholerae* 0395 was calculated as follows:

Table 2.2. Example dataset for LD₅₀ calculation.

Dose	$N_1 \times 10^7$	$N_2 \times 10^6$	$N_3 \times 10^5$
Dead/Live pups	D_7/L_7	D_6/L_6	D_5/L_5
Total dead pups	$D_1 = D_7 + D_6 + D_5$	$D_2 = D_6 + D_5$	$D_3 = D_5$
Total live pups	$L_1 = L_7$	$L_2 = L_7 + L_6$	$L_3 = L_7 + L_6 + L_5$
Proportion = $\frac{\text{Total dead pups}}{\text{Total pups}}$	$P_1 =$ $\frac{D_1}{D_1 + L_1}$	$P_2 =$ $\frac{D_2}{D_2 + L_2}$	$P_3 =$ $\frac{D_3}{D_3 + L_3}$

Algorithm for calculating LD₅₀:

1. From table 2.2 let $P_1 (> 0.5)$ and $P_2 (< 0.5)$ be the proportions that bracket 0.5 (by inspection).
2. Let $\Delta P = P_1 - P_2$
3. Let $\delta P_2 = 0.5 - P_2$
3. Let $R = \frac{\delta P_2}{\Delta P}$
4. Let (from Table 2.2) $N_i \times 10^j$ be the lower bracket dose
5. Let $S = R + j$
6. Let $T = \text{antilog}(S)$
7. Let $LD_{50} = N_i \times T$

In some cases, small intestines from the pups were recovered at 48 hr post-inoculation and homogenized in 1 ml of PBS using Dounce homogenizers. A dilution series was prepared from this homogenate and 100 μ l of the appropriate dilutions were plated on L agar supplemented with 67 μ g/ml of streptomycin sulfate. *V. cholerae* harvested from the small intestines was calculated as CFU/ml using equation 2.1.

Results

3.1 Conversion of MMTV to a Lymphomagenic Virus

3.1.1 Overview

TBLV is an MMTV variant that causes thymic lymphomas, instead of mammary tumors, in mice. Unlike the MMTV LTR, the TBLV LTR has a 443 bp deletion that eliminates the NREs and duplicates NRE-flanking sequences to generate a T-cell enhancer. The deletion and substitution also truncate the TBLV *sag* gene. Substituting a portion of the MMTV LTR U3 region in an infectious MMTV clone (HYB-MTV) with a 438 bp segment (encompassing the deletion and substitution) of the TBLV LTR produces a recombinant virus that induces thymic lymphomas instead of mammary tumors in a *Sag*-independent manner. To evaluate the role of *cis*-acting LTR elements (NREs and the T-cell enhancer) in converting MMTV to a lymphomagenic virus, mutant HYB-MTV proviruses were constructed by inserting the TBLV T-cell enhancer in the 3' LTR either at the *Stu*I site upstream of the NRE (HYB-MTV*Stu*E) or at the *Afl*III site downstream of the NRE (HYB-MTV*Afl*E). A third mutant provirus (HYB-MTV Δ NRE) lacking the entire NRE

and the flanking T-cell enhancer sequences in the 3' LTR was also constructed. Weanling BALB/cJ mice inoculated with HYB-MTV Δ NRE developed a low frequency of mammary tumors, while HYB-MTV Δ flE and HYB-MTV Δ StuE had similar incidences of both mammary tumors and thymic lymphomas. Expression of the T-cell enhancer in proviruses from HYB-MTV Δ flE and HYB-MTV Δ StuE-induced lymphomas was confirmed by RT-PCR. In contrast, Southern analysis of proviruses integrated in the lymphomas revealed that the NRE was retained in HYB-MTV Δ flE proviruses, but deleted from HYB-MTV Δ StuE proviruses. Transient transfection assays in T cells demonstrated that the T-cell enhancer completely masked the NRE activity when located downstream of the NRE (HYB-MTV Δ flE), yet had only a partial effect when placed upstream of the NRE (MTV Δ StuE LTR). Thus, conversion of MMTV to a lymphomagenic virus requires two alterations in the LTR - (i) acquisition of a T-cell specific enhancer and (ii) loss of NRE-mediated transcriptional repression through either NRE deletion or by masking the NRE activity via a T-cell enhancer.

3.1.2 TBLV lymphomagenesis is Sag-independent.

Yanagawa *et al.* [475] demonstrated that the tropism of the MMTV hybrid provirus, HYB-MTV [403], could be altered by replacement of its 3' LTR with that from thymotropic MMTVs, DL-8 and MA. The resulting recombinant viruses, DL-8 MMTV and MA MMTV, induced thymic lymphomas in 20 and 29% of adult BALB/cJ mice with average latencies of 26 and 34 weeks, respectively. Although this study established the critical role of the LTR in determining viral tumorigenesis,

the contributions of the truncated Sag and *cis*-acting elements in the LTR relative to the tissue specificity of tumorigenesis was not determined.

Therefore, TBLV-specific sequences from the *Cla*I to *Sst*I sites in the LTR (438 bp), encompassing the NRE deletion and T-cell enhancer substitution [27, 286], were used to replace 756 bp from the *Cla*I to *Sst*I region of the pHYB-MTV 3' LTR. This molecular clone has been called pHYB-TBLV (Figure 3.1) [317]. Frameshift mutations were introduced at the *Avr*II and *Cla*I sites in the truncated *sag* gene of pHYB-TBLV to generate the *sag*-frameshift mutant, pHYB-TBLV*sag*DFS (Figure 3.1). The frameshift mutations further truncate the *sag* gene in pHYB-TBLV*sag*DFS to encode only the first 17 amino acids of Sag.

The frameshift mutations in pHYB-TBLV*sag*DFS are located within the transcriptional regulatory sequences in the 3' LTR. Therefore, the 3' LTRs from pHYB-TBLV*sag*DFS and pHYB-TBLV were inserted into a promoterless firefly luciferase (LUC) expression vector [444] and transiently transfected into Jurkat T cells. The LUC activity from the two LTRs did not differ significantly (Figure 3.2) suggesting that the frameshift mutations did not alter the LTR transcriptional efficiency.

Subsequently, Jurkat T cells were stably transfected with either wild-type or mutant hybrid TBLV proviruses (Jurkat/HYB-TBLV and Jurkat/HYB-TBLV*sag*DFS). Jurkat T cells were chosen because of their high efficiency of transfection and because the presence of the TBLV enhancer should induce high levels of viral expression, thus allowing a high efficiency of infection and tumorigenesis following inoculation into mice. Transfected cells then were tested

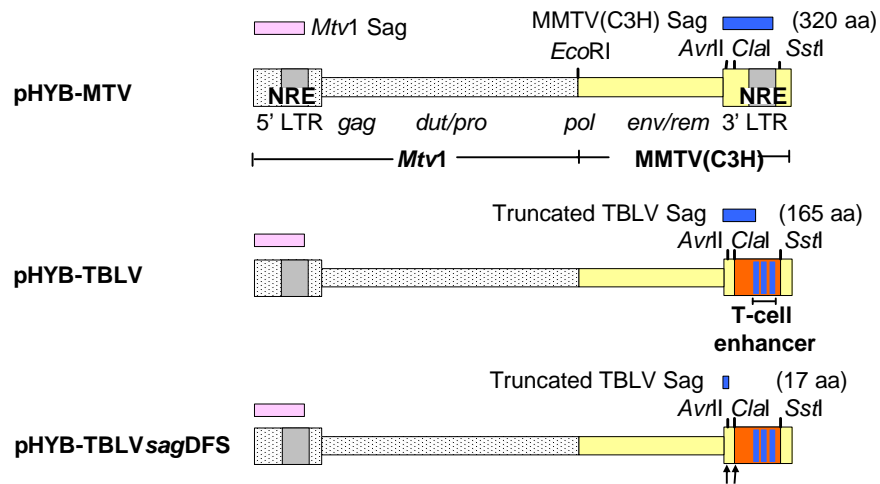


Figure 3.1. Construction of hybrid MMTV-TBLV proviral molecular clones.

The pHYB-MTV plasmid [403] contains a hybrid MMTV provirus whose 5' half (stippled) upstream of the *EcoRI* site is derived from endogenous *Mtv1* and the 3' half (yellow) downstream of the *EcoRI* site is derived from exogenous MMTV(C3H). The approximate positions of viral genes are shown, and the LTRs are represented by the larger boxes at the ends of the provirus. The grey boxes within the MMTV LTRs represent the NRE. The Sag proteins are designated by boxes above the proviral constructs. pHYB-TBLV was constructed by replacing the *ClaI* to *SstI* region of the 3' LTR of pHYB-MTV with the corresponding section (orange box) from the TBLV LTR that encompasses the NRE deletion and triplicate T-cell enhancer (blue bars) substitution. A mutant TBLV hybrid provirus, pHYB-TBLVsagDFS, was constructed by introducing frameshift mutations at the *AvrII* and *ClaI* sites in the *sag* coding sequence (indicated by arrows). These mutations allow translation of only 17 amino acids of Sag.

for expression of MMTV capsid protein by Western blotting using MMTV CA-specific monoclonal antibodies [350]. Comparable levels of Gag protein expression were detected in the cell lysates (Figure 3.3A) as well as in the concentrated culture supernatants (Figure 3.3B) from the two transfected cell lines.

The Jurkat/HYB-TBLV or Jurkat/HYB-TBLVsagDFS cells were inoculated intraperitoneally into weanling BALB/cJ mice. Thymic lymphomas developed in 50% of the HYB-TBLV infected mice with an average latency of 5.7 (\pm 1.5)

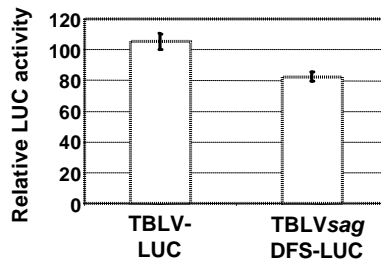


Figure 3.2. TBLV and TBLVsagDFS LTRs demonstrate similar transcriptional efficiencies in transiently transfected Jurkat T cells.

Transcriptional efficiencies of wild-type (TBLV) and mutant (TBLVsagDFS) LTRs in Jurkat T cells were compared by transient transfection of LTR-driven LUC reporter plasmids. LUC activity is reported in light units normalized for DNA uptake as measured by co-transfection with the *Renilla* luciferase expression plasmid, pRL-TK. LUC activity from the pTBLVsagDFS-LUC plasmid is depicted relative to that from the pTBLV-LUC plasmid (assigned a value of 100). Standard deviations from the means of triplicate assays are shown.

months, whereas HYB-TBLVsagDFS-infected mice developed T-cell lymphomas with a 60% incidence and an average latency of 4.8 (\pm 0.6) months (Table 3.1) [317]. Statistical analysis did not reveal any significant difference in the incidence and latency of tumor induction by the wild-type and Sag-mutant TBLV ($P > 0.05$). Interestingly, one mouse infected with HYB-TBLVsagDFS developed a mammary tumor with a latency of 9.5 months (Table 3.1). Since this was the only mammary tumor observed in the study, the viral origin of the tumor was not determined.

Expression of HYB-TBLV or the *sag* frameshift mutant virus in these tumors was confirmed using RT-PCR followed by sequencing (data not shown). RT-PCR analysis also detected three enhancer elements in the U3 region of viral RNA expressed in most of these lymphomas, while variants containing one or two enhancer elements were also present at low levels (Figure 3.4).

These results revealed that substitution of the 438 bp *Cla*I to *Sst*I region

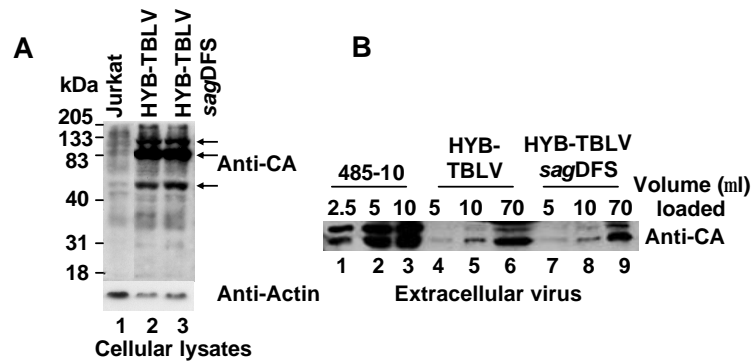


Figure 3.3. Jurkat T cells stably transfected with proviral constructs express viral proteins and release extracellular virions.

(A) Western blot analysis of whole cell lysates from Jurkat T cells stably transfected with pHYB-TBLV or pHYB-TBLV*sagDFS*. Whole cell lysates prepared from untransfected Jurkat cells were used as a negative control. Each lane was loaded with 100 μ g of cellular lysate. Viral Gag expression was detected using MMTV CA-specific antibody (upper panel). Arrows indicate processed and unprocessed forms of Gag. Fifty micrograms of each cellular lysate was analyzed by Western blotting using actin-specific antibodies (lower panel) as a control for protein loading. (B) Extracellular virus production from Jurkat T cells stably transfected with pHYB-TBLV or pHYB-TBLV*sagDFS*. Varying amounts of cell culture supernatants from the transfected cells were analyzed by Western blotting using MMTV CA-specific antibody and compared to culture supernatant obtained from the TBLV-induced lymphoma, 485-10.

from the TBLV LTR into the 3' LTR of pHYB-MTV is sufficient to alter MMTV disease tropism and create a lymphomagenic virus. Furthermore, these results also established that the truncated TBLV Sag is not essential for the development of virally-induced thymic lymphomas in the BALB/cJ strain.

3.1.3 TBLV and TBLV*sagDFS*-induced tumors have similar phenotypes.

To compare the phenotypes of T-cell lymphomas induced by the wild-type (HYB-TBLV) and the mutated (HYB-TBLV*sagDFS*) viruses, the tumor cell populations were labeled for cell surface markers and analyzed using flow

Table 3.1. Incidence and latency of tumors induced by clonal TBLV proviruses after injection of transfected Jurkat T cells.

Hybrid provirus	Tumor type induced	Number of mice injected (F/M) ^a	Incidence of tumors (%)	Average tumor latency (months)	Duration of observation (months)
HYB-TBLV	T-cell lymphoma	12/6	50	5.7 ± 1.5 ^b	12
HYB-TBLV <i>sag</i> DFS	T-cell lymphoma	22/8	60	4.8 ± 0.6 ^b	12
	Mammary tumor		4.6 ^c	9.5 ^d	

^a Jurkat T-cell transfectants (2×10^7 cells) were injected intraperitoneally into the indicated number of female (F) and male (M) weanling BALB/cJ mice.

^b There was no statistically significant difference ($P = 0.08$) between the incidence and average latencies of tumors induced by the wild-type and *sag* double-frameshift mutant viruses. Differences between wild type and mutant-injected animals were analyzed using the Statistical Package for Social Sciences (SPSS) (SPSS, Inc., Chicago, IL.). Time to tumor appearance was evaluated using the Kaplan-Meier survival analysis technique with a log-rank test of significance. This analysis excluded one HYB-TBLV-induced tumor that appeared at 12 months.

^c The mammary tumor incidence was based on the number of female mice injected.

^d Only one mammary tumor was observed.

cytometry. Most tumors had a high percentage of Thy1.2+ cells, confirming that the lymphomas had originated from the host T-cell lineage (Table 3.2). The Thy1.2+ tumor cell populations were heterogeneous with respect to the surface distribution of CD4 and CD8 markers, and there was no significant difference between the two groups. The tumors were not outgrowths of the originally injected cells since the antibodies used for cell surface staining did not react with Jurkat cells (data not

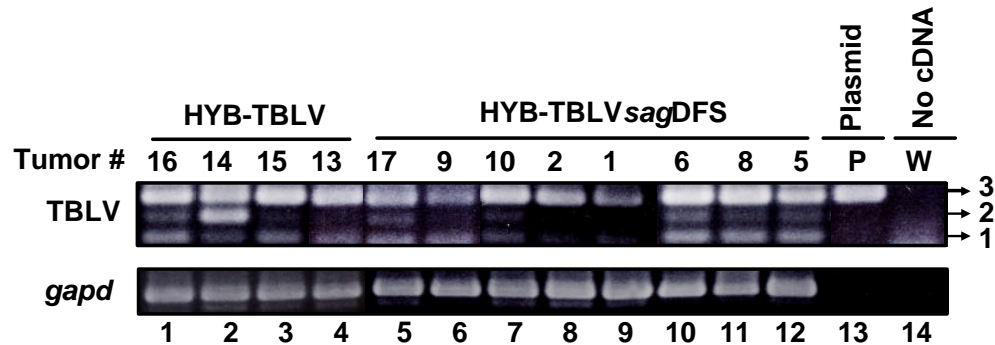


Figure 3.4. RT-PCR analysis of HYB-TBLV and HYB-TBLV_{sag}DFS-induced lymphomas.

RT-PCR analysis of the number of enhancer elements (upper panel) in the U3 region of the viral RNA expressed in the thymic lymphomas was performed using primers C3HLTR501(+) and TBLVLTR786(-). The amplified products obtained from viral RNA U3 regions containing 1, 2, and 3 enhancer elements are indicated by arrows. Tumors 13, 14, 15, and 16 were induced by HYB-TBLV while tumors 1, 2, 5, 6, 8, 9, 10, and 17 were induced by HYB-TBLV_{sag}DFS. RT-PCR amplification of *gapd* mRNA (lower panel) was performed as a control for RNA integrity.

shown).

To analyze the clonality of thymic lymphomas induced by wild-type and *sag*-frameshift TBLV, TCR β and γ chain rearrangements were analyzed by Southern blotting (Figure 3.5). Most of the tumors induced by either virus showed rearrangement of both TCR chains, and many tumors had a significant clonal population. These results combined with the cell surface analysis suggested that the lymphomas induced by HYB-TBLV and HYB-TBLV_{sag}DFS were phenotypically similar and were of oligoclonal origin. The tumor phenotypes were also similar to those of tumors induced by intrathymic injection of newborn mice with non-clonal TBLV virions produced by the lymphoma line, 485-10 [309, 310].

Thus, a novel TBLV tumorigenicity assay in adult mice was developed using a cloned hybrid TBLV provirus and used to evaluate the role of TBLV truncated Sag

Table 3.2. Comparison of cell surface markers on lymphomas induced by HYB-TBLV and HYB-TBLV_{sagDFS} in adult BALB/cJ mice.

Hybrid provirus	Tumor number	% Thy1.2+ CD4+	% Thy1.2+ CD8+	% Thy1.2+ CD4- CD8-	% Thy1.2+ CD4+ CD8+
HYB-TBLV ^a	11	9	2.1	2	87
	12	73	0.8	25	1.7
	13	27	0.6	72	0.1
	14	4.1	22	2.4	71
	15	26	0.6	3	70
HYB-TBLV _{sagDFS} ^a	1	21	45	13	22
	2	3.4	8.3	2.6	86
	3	19	3.8	5.5	72
	4	5.7	11.4	4.4	79
	5	74	0.24	24	1.5
Normal BALB/cJ thymus ^b		11	3.3	2.1	84

^a Statistical analysis was performed using *t*-tests with and without logarithm transformation to stabilize the variance.

^b The numbers represent average values obtained from 10 weanling BALB/cJ mice.

in determining lymphomagenicity. Our results demonstrated that the truncated Sag is dispensable for TBLV tumorigenicity, suggesting that MMTV disease specificity is determined by the activity of *cis*-acting elements in the viral LTR.

3.1.4 TBLV enhancer masks the MMTV NRE activity.

To determine the contributions of the NRE and the T-cell enhancer on TBLV lymphomagenicity, two mutant MMTV LTRs were engineered by inserting the TBLV T-cell enhancer (E) either at the *StuI* site upstream of the NRE (MTV*StuE* LTR) or at the *AflII* site downstream of the NRE (MTV*AflE* LTR) leaving the

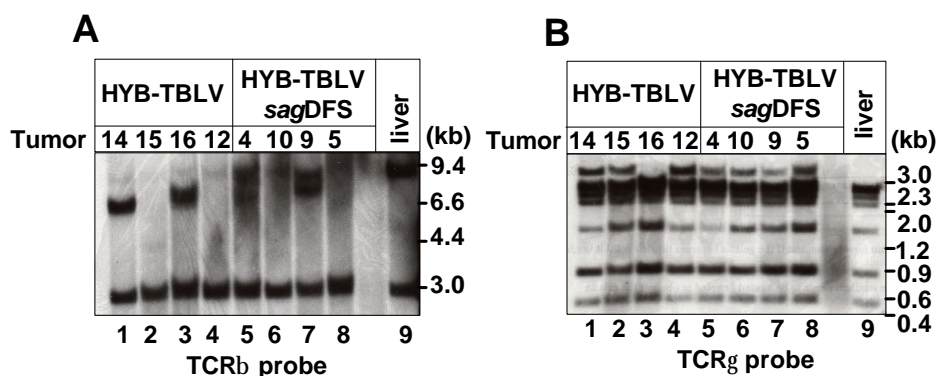


Figure 3.5. Analysis of TCR gene rearrangements in HYB-TBLV and HYB-TBLV $sagDFS$ -induced lymphomas.

(A) Southern blotting for TCR β chain rearrangements. (B) Southern blotting for TCR γ chain rearrangements. Tumors 12, 14, 15, and 16 were induced by HYB-TBLV, whereas tumors 4, 5, 9, and 10 were induced by HYB-TBLV $sagDFS$. Digestion of liver DNA from uninfected animals was used to demonstrate the DNA fragments derived from the unrearranged TCR genes. Genomic DNA (15 μ g) was digested with *Hind*III, separated on 0.8% agarose gels, transferred to nitrocellulose and hybridized with probes p86T5 β (for TCR β chain) [125] or C γ 1.2 (for TCR γ chain) [208].

NRE intact in both LTRs (Figure 3.6A). While the *sag* coding sequence remained unaffected in the MTVA β E LTR, enhancer insertion in the MTV Stu E LTR truncated the *sag* gene at a position similar to that in the TBLV LTR [27]. The third recombinant LTR, MTV Δ NRE, used in this study has been described previously [286]. This recombinant lacks the entire NRE and the flanking T-cell enhancer sequences (Figure 3.6A).

The MTV Δ NRE LTR demonstrated a 3-fold elevated transcriptional efficiency compared to the MMTV LTR in Jurkat T cells transiently transfected with LTR-driven firefly luciferase (LUC) reporter plasmids [286, 444]. However, the TBLV T-cell enhancer located upstream of the NRE partially relieved NRE activity, resulting in a 250-fold increased LUC expression from the MTV Stu E LTR relative

to the MMTV LTR (Figure 3.6B). MTVA/*fE* and TBLV LTRs demonstrated similar transcriptional strengths, achieving 700-fold elevated LUC expression compared to the MMTV LTR (Figure 3.6B). These data indicate that the T-cell enhancer placed between the MMTV NRE and the LTR promoter completely masks NRE activity in transiently transfected Jurkat T cells.

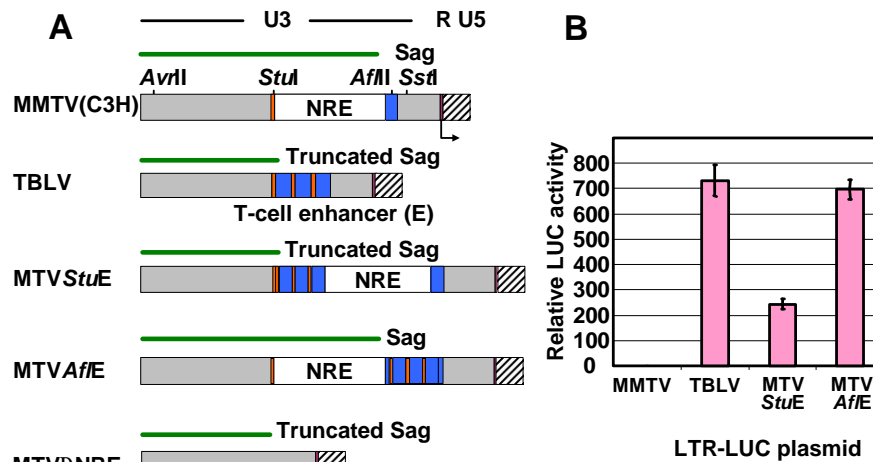


Figure 3.6. Construction and characterization of mutant MMTV LTRs.

(A) Diagram depicting the composition of MMTV, TBLV and mutant MMTV LTRs. The arrow on the MMTV LTR denotes the transcription start site at the U3-R border. The *sag* coding region is depicted as green lines above each LTR. However, Sag is not expressed in these assays. (B) Transient transfection assay comparing the transcriptional efficiencies of wild-type MMTV and TBLV LTRs and their recombinants in the Jurkat T-cell line. LUC activity is reported in light units normalized for DNA uptake as measured by co-transfection with the *Renilla* luciferase expression plasmid, pRL-TK. LUC activity from the TBLV and mutant MMTV LTRs is depicted relative to that from the MMTV LTR (assigned a value of 1). Standard deviations from the means of triplicate assays are shown.

3.1.5 TBLV enhancer improves glucocorticoid induction of MMTV LTR.

In the absence of glucocorticoid [dexamethasone (DEX)] the MTV Δ fE, MMTV, TBLV, and MTV Δ StuE LTRs exhibited statistically similar transcriptional efficiencies in transient transfections of the HC11 mouse mammary gland cell line, indicating the lack of a significant transcriptional advantage from T-cell enhancer addition [286] (Figure 3.7). Under the same conditions, the MTV Δ NRE LTR had a higher transcriptional activity compared to the MMTV LTR (P=0.03) (Figure 3.7). In the presence of DEX, transcription from the MMTV LTR, which harbors 6 HREs [141], increased five-fold (P=0.005). The MTV Δ NRE LTR with 3 HREs did not undergo a statistically significant DEX induction (P=0.2). The T-cell enhancer (containing a triplicated HRE) provided a transcriptional advantage in the presence of DEX since the transcriptional efficiencies of TBLV and MTV Δ fE LTRs demonstrated ca. 30-fold DEX-induction compared to the uninduced MMTV LTR (P=0.01 and <0.001, respectively) (Figure 3.7). However, DEX responsiveness of the MTV Δ StuE LTR was significantly lower than that of the MTV Δ fE and TBLV LTRs (P=0.001 and 0.01, respectively), suggesting that positioning the NRE between the enhancer and the promoter mitigates the DEX-responsive transcriptional influence of the T-cell enhancer.

3.1.6 Lymphomagenic MMTVs require a T-cell enhancer and loss of the NRE activity.

Recombinant MMTV LTRs were prepared by substitution of *Avr*II to *Sst*I fragments for the corresponding region of pHYB-MTV 3' LTR [403]. Jurkat T

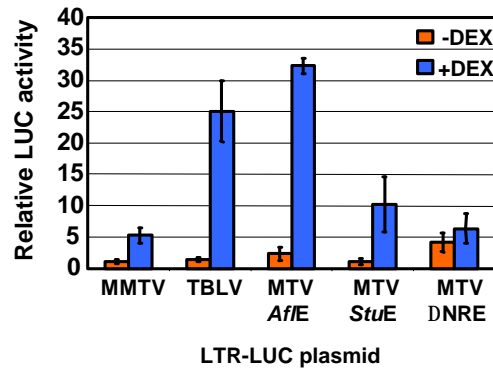


Figure 3.7. Transient transfection assay comparing the transcriptional efficiencies of wild-type and mutant MMTV and TBLV LTRs in HC11 cells.

Transfection assays were performed in HC11 mouse mammary gland cell line in the absence (orange bars) or presence (blue bars) of dexamethasone (DEX). LUC activity is reported in light units normalized for DNA uptake as measured by co-transfection with the pRL-TK reporter plasmid. LUC activity from the TBLV and mutant MMTV LTRs is depicted relative to that from the MMTV LTR in the absence of DEX (assigned a value of 1). Standard deviations from the means of triplicate assays are shown. The results were analyzed by pair-wise *t*-tests.

cells that stably express wild-type (HYB-MTV) or recombinant viruses (HYB-MTV $StuE$, HYB-MTV A/E and HYB-MTV ΔNRE) were derived. Comparable viral Gag expression in all the stable cell lines was verified by Western blot analysis of whole cell lysates (Figure 3.8) using MMTV CA-specific monoclonal antibody [350]. Weanling BALB/cJ mice were intraperitoneally inoculated with 2×10^7 virus-expressing cells, and viral infection was followed by flow cytometry analysis of peripheral Sag-reactive CD4⁺ T cells.

Of the peripheral, Sag-reactive CD4⁺V β 14⁺ T cells, 37 to 59% were deleted at 3 months post-inoculation with Jurkat/HYB-MTV and Jurkat/HYB-MTV A/E , respectively (Figure 3.9), whereas non-reactive CD4⁺V β 8⁺ T cells remained unaffected. Sag-reactive T cells were within the normal range (8 to 10%)

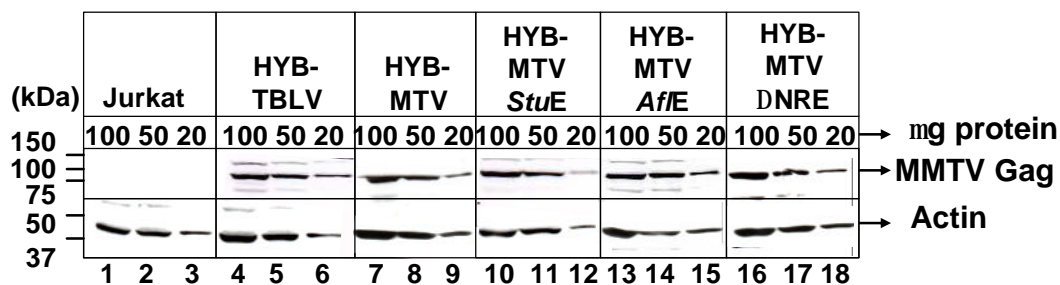


Figure 3.8. Characterization of mutant MMTV proviruses.

Western blot analysis comparing viral Gag expression in Jurkat T cells stably expressing wild type (HYB-MTV) or mutant (HYB-MTV*StuE*, HYB-MTV*AffE* and HYB-MTV Δ NRE) MMTV proviruses. Three different amounts (100, 50 and 20 μ g) of whole cell lysates were analyzed on 8% SDS-PAGE gels. Whole cell lysates derived from untransfected Jurkat T cells were included as a negative control. Viral Gag (upper panel) was detected with an MMTV CA-specific monoclonal antibody [350]. The arrow indicates the precursor form of Gag, Pr77. The same amounts of cellular lysates were incubated with antibodies specific for actin (lower panel) as a control for protein loading on the gel.

in Jurkat/HYB-MTV*StuE* infected mice, confirming the inability of the truncated Sag to mediate T-cell deletion.

HYB-MTV induced mammary tumors with a 100% incidence and average latency of 7.3 ± 1.7 months (Figure 3.10). HYB-MTV Δ NRE also induced only mammary tumors in 20% of the inoculated female mice within 10 ± 2.8 months indicating that LTR NRE deletion alone was insufficient for lymphomagenesis. In contrast, 50% of the HYB-MTV*StuE*-infected mice developed mammary tumors within 8.7 ± 2.1 months, while 30% developed thymic lymphomas within 8 ± 2.6 months. One HYB-MTV*StuE*-infected mouse (mouse no. 4) developed both tumor types. HYB-MTV*AffE* infection gave a 17% incidence of mammary tumors and a 22% incidence of thymic lymphomas with average latencies of 6 and 6.8 ± 1.1 months, respectively. Kaplan-Meier plots followed by Wilcoxon

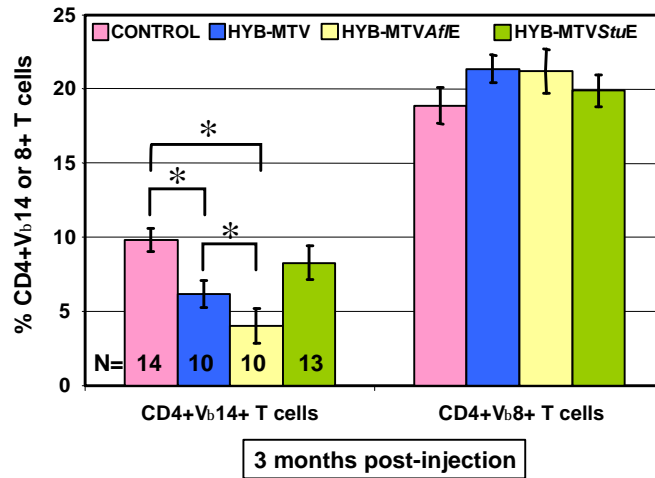


Figure 3.9. Peripheral Sag-reactive CD4+ T cells are deleted in BALB/cJ mice inoculated with Jurkat T cells stably expressing full-length Sag-encoding proviruses.

Flow cytometric analysis of Sag-mediated peripheral T-cell deletion in mice at 3 months post-injection with Jurkat T cells stably expressing wild-type or mutant MMTV proviruses. Sag-reactive CD4+Vβ14+ and Sag-non-reactive CD4+Vβ8+ peripheral T cells were analyzed on the FACSCalibur using CELLQuest software. The percentage of TCR Vβ+ cells in the gated CD4+ T-cell population was calculated. The number of animals (N) analyzed from each group is indicated within each bar. The mean percentage (\pm standard deviation) of CD4+TCR Vβ+ T cells in each group is shown. Results were analyzed by pair-wise *t*-tests. The asterisks indicate a significant difference ($P<0.05$) between the indicated pairs.

or log-rank tests indicated that the mammary tumor incidence of each of the mutants was significantly different than that of wild-type HYB-MTV (Figure 3.10A). Mammary tumor incidence was not statistically different among the various mutants. Lymphomas have never been observed after infection with HYB-MTV (Figure 3.10B), even after inoculation of much larger numbers of animals [466, 484].

The LTR structure of newly integrated proviruses was then determined by Southern blot analysis of *Pst*I digested tumor genomic DNA (Figure 3.11)

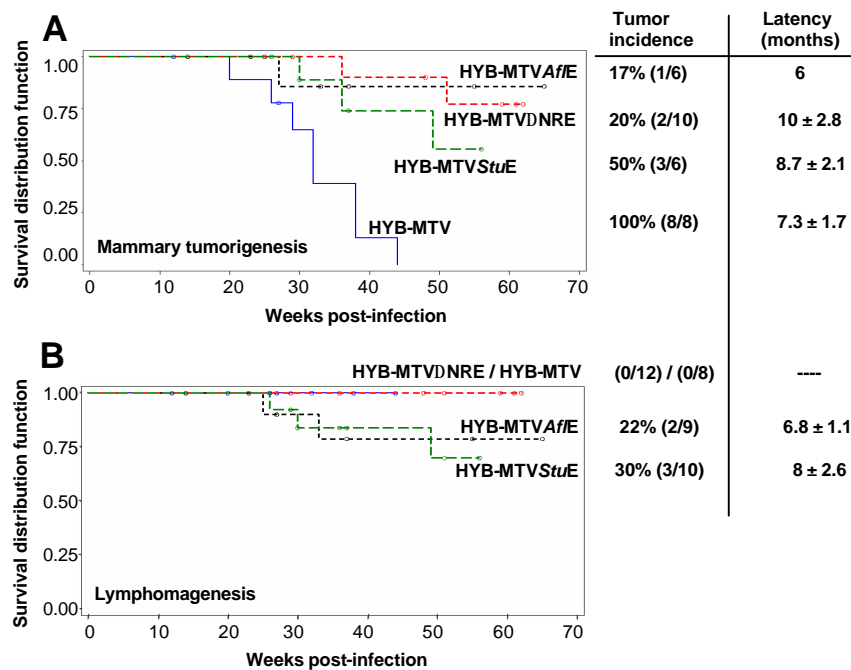


Figure 3.10. Kaplan-Meier plots of mammary tumors and thymic lymphomas induced by wild-type and mutant MMTV proviruses.

Females were caged to maintain continuous breeding and lactation. Mice that died from non-tumor causes (e.g., birthing problems) before the average latency of tumor induction were censored from the study. (A) Development of mammary tumors. Numbers in parentheses indicate the number of animals with tumor/total number of injected females. The circles indicate one or more animals that died without any detectable tumors. (B) Development of thymic lymphomas. Numbers in parentheses indicate number of animals with tumor/total number of injected males and females. One mouse injected with HYB-MTV*StuE* developed both a mammary tumor and a thymic lymphoma after 11 months of latency.

and either MMTV LTR (Figure 3.12A) or NRE-specific probes (Figure 3.12B). The LTRs of predominant newly integrated proviruses in HYB-MTVAf/E-induced lymphomas contained the NRE (Figure 3.12A-B, lanes 5 and 7). The majority of these LTRs retained their original size suggesting an intact triplicate T-cell enhancer, while smaller sized LTRs might harbor fewer enhancer copies or partial

NRE deletion. TBLV-induced lymphomas reportedly select integrations with suboptimal number of enhancer repeats to modulate *c-myc* expression [59]. LTRs of predominant newly integrated proviruses in HYB-MTV*StuE*-induced lymphomas lacked the entire NRE and were detected only by the MMTV LTR probe (Figure 3.12A-B, lanes 10 and 11). The size reduction of these LTRs was consistent with complete NRE deletion in the presence of the T-cell enhancer. NRE-minus recombinants were not observed in HYB-MTV*StuE*-induced mammary tumors (Figure 3.12A-B, lanes 8, 9 and 12). Interestingly, the LTRs of newly integrated proviruses in the HYB-MTV Δ NRE-induced mammary tumor had reacquired the NRE, suggesting that the presence of this region might favor mammary tumorigenesis (Figure 3.12A-B, lane 13).

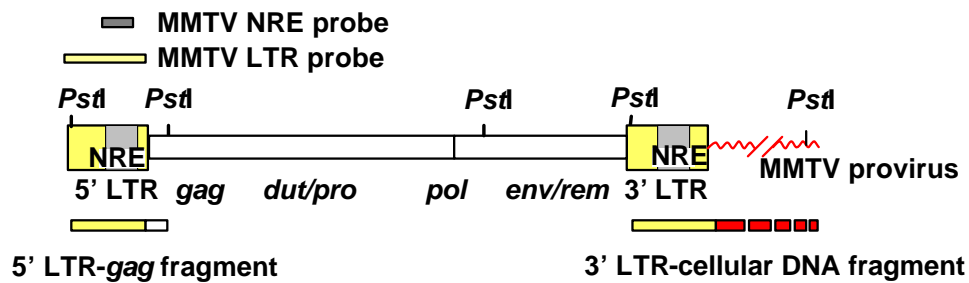


Figure 3.11. Strategy for analyzing the LTR structure of newly integrated MMTV proviruses in the wild-type and mutant MMTV-induced tumors.

Tumor genomic DNA digested with *Pst*I, separated on 0.8% agarose gels, and transferred onto nitrocellulose membranes were hybridized to probes derived from the MMTV LTR or only from the NRE region of the MMTV LTR. *Pst*I yields four fragments from newly integrated exogenous MMTV proviruses, only two of which are detectable by the MMTV LTR and NRE probes. (i) The 5' LTR-gag fragment whose size is determined by the LTR length and, (ii) the 3' LTR-cellular DNA fragment whose size is dependent on the length of the LTR and the flanking cellular DNA. Since the LTR probe will detect fragments originating from all MMTV proviruses, the MMTV NRE-specific probe will only detect fragments from proviruses that contain the NRE in their LTRs.

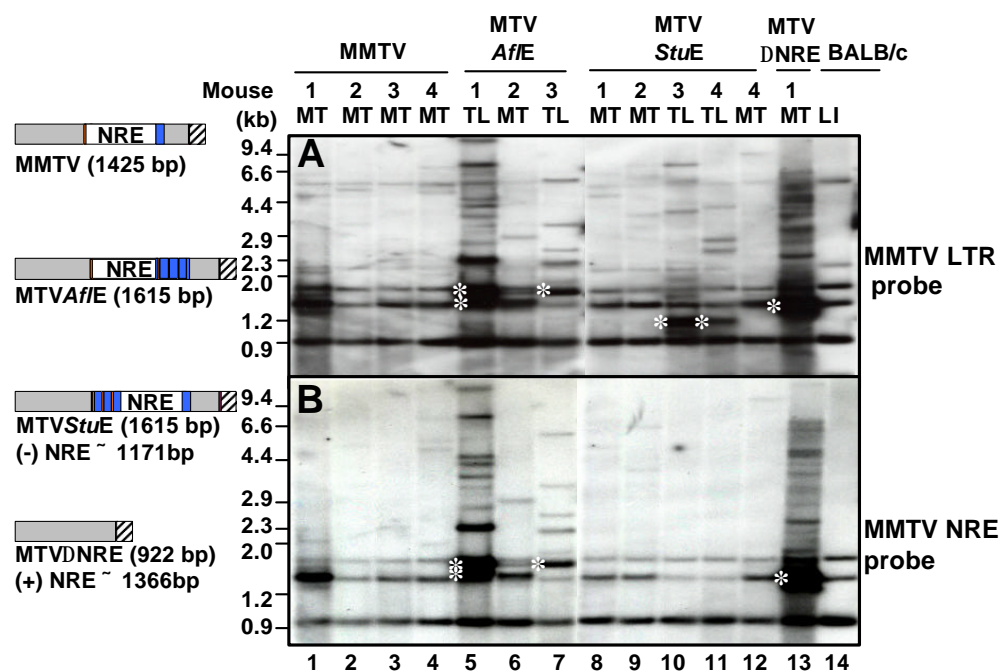


Figure 3.12. Analysis of the LTR structure of newly integrated proviruses in wild-type and mutant MMTV-induced mammary tumors (MT) and thymic lymphomas (TL).

(A) Southern blotting using the whole MMTV LTR as the probe. (B) Southern blotting with MMTV NRE probe (*Stu*I to *Afl*III fragment of the MMTV LTR). Southern blotting of genomic DNA from an uninfected BALB/cJ mouse liver (LI) was used to distinguish the bands originating from the three endogenous *Mtvs* in the BALB/cJ strain. White asterisks placed in lanes 5, 7, 10, 11, and 13 indicate the LTR fragments of predominant newly integrated proviruses in tumor DNA.

The tumors were also analyzed by RT-PCR and the products were gel-purified and sequenced or, in some cases, cloned into pGEM[®]-T Easy vector (Promega) prior to sequencing (Figure 3.13). Most of the U3 sequences amplified from viral RNA expressed in HYB-MTV*Stu*E-induced lymphomas retained two to three copies of the T-cell enhancer, yet lacked the entire NRE. Some amplified U3 sequences had lost the T-cell enhancer while retaining the NRE. However, proviruses containing these LTR U3 regions were not predominant in the tumor

genomic DNA as revealed by Southern blotting (Figure 3.12). LTRs of predominant newly integrated proviruses in the HYB-MTV*StuE*-induced lymphomas were undetectable by the NRE probe and their size was consistent with the loss of the entire NRE and retention of the T-cell enhancer.

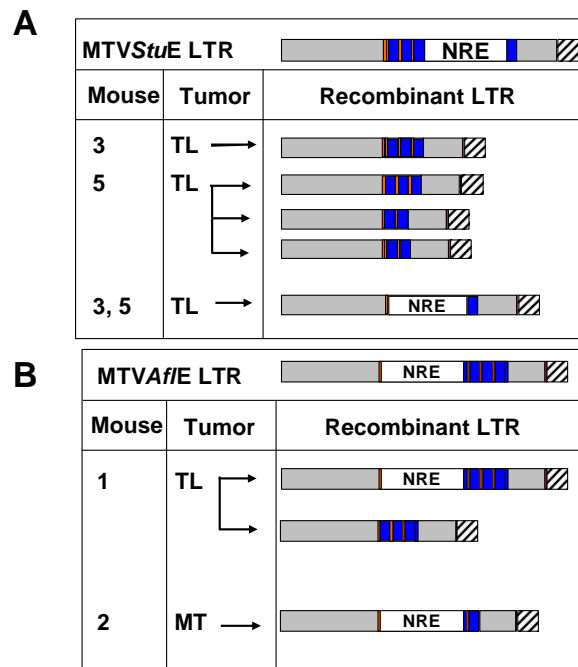


Figure 3.13. RT-PCR analysis of the LTR composition of MMTV proviruses expressed in mutant MMTV-induced tumors.

(A) LTR composition of MMTV proviruses expressed in HYB-MTV*StuE*-induced thymic lymphomas (TL). (B) LTR composition of MMTV proviruses expressed in HYB-MTV*AfE*-induced thymic lymphomas (TL) and mammary tumors (MT).

Similar RT-PCR analysis of HYB-MTV*AfE*-induced lymphomas (Figure 3.13) revealed the presence of both the NRE and the TBLV enhancer in the viral U3 sequences. Some amplified U3 sequences had lost the NRE and retained only the T-cell enhancer, but proviruses containing these LTR U3 regions were not

predominant in the tumor genomic DNA as determined by Southern blotting (Figure 3.12). LTRs of the predominant newly integrated proviruses in the HYB-MTV Δ flE-induced lymphomas were detected by the NRE probe, and their size was consistent with the presence of the entire NRE and three (or fewer at a lower frequency) copies of enhancer elements. In contrast, the predominantly expressed proviruses in the HYB-MTV Δ flE and HYB-MTV Δ StuE-induced mammary tumors had lost the T-cell enhancer while retaining the NREs in their LTRs. This observation was also reinforced by Southern blot analysis of mammary tumors that revealed the predominance of proviruses with smaller LTRs detectable by the MMTV NRE probe. The reduced size of these LTRs correlated with the length of the T-cell enhancer.

These results demonstrated that conversion of the mammotropic MMTV to a lymphomagenic virus requires T-cell enhancer acquisition and loss of NRE activity achieved either by NRE deletion or by masking the NRE activity by a downstream enhancer. These results also revealed that truncation of the *sag*-coding sequence is not a pre-requisite for TBLV-induced lymphomagenesis since the HYB-MTV Δ flE virus that retained full-length Sag coding potential was lymphomagenic.

3.2 An *Mtv*-Free BALB/cJ Congenic Strain is Resistant to MMTV, TBLV and *Vibrio cholerae*.

3.2.1 Overview

Most laboratory mouse strains harbor endogenous MMTV proviruses (*Mtvs*) in their germline. Previous data have suggested that endogenous *Mtvs* may protect against infection by milk-borne MMTVs with the same Sag specificity. An endogenous *Mtv*-free, BALB/cJ congenic mouse strain (BALB/*Mtv*-null), which develops low-grade MMTV(C3H) infection and demonstrates only a 5 to 10% incidence of mammary tumors was developed. Moreover, these mice fail to undergo Sag-reactive T-cell deletion. In contrast, BALB/cJ mice develop a 90 to 100% incidence of MMTV(C3H)-induced mammary tumors and also demonstrate robust Sag-mediated T-cell deletion. BALB/*Mtv*-null mice are also totally resistant to Sag-independent TBLV lymphomagenesis in contrast to BALB/cJ mice, which develop a 50% incidence of TBLV-induced thymic lymphomas. Genetic studies demonstrate that resistance to MMTV-induced tumors in BALB/*Mtv*-null mice is a recessive trait linked to the absence of endogenous *Mtvs*. BALB/cJ congenic strains (BALB/*Mtv*-SP) harboring any one of the three endogenous *Mtvs* (*Mtv*6, 8 or 9) located on three separate chromosomes of the BALB/cJ strain are susceptible to MMTV tumorigenesis and Sag-mediated T-cell deletion. Production of MMTV neutralizing antibodies does not contribute to the resistance trait since only about 30% of the MMTV(C3H)-infected BALB/*Mtv*-null mice develop a low titer of MMTV capsid-specific antibodies. The BALB/*Mtv*-null strain is also more resistant

to disease induced by the bacterial pathogen, *Vibrio cholerae*, which exhibits an LD₅₀ of 2.3×10^6 CFU in this strain. In contrast, *V. cholerae* exhibits a 10-fold lower LD₅₀ (1.6×10^5 CFU) in the BALB/cJ strain. Interestingly, the *V. cholerae* LD₅₀ for all three BALB/*Mtv*-SP strains ranges between 1 to 3×10^5 CFU, suggesting that the heightened susceptibility to *V. cholerae* also co-segregates with each of the three endogenous *Mtvs* of the BALB/cJ strain. The absence of endogenous *Mtvs* might allow an altered immune response that forms the basis of a novel mechanism of resistance to both viral and bacterial pathogens.

3.2.2 BALB/cJ congenic BALB/*Mtv*-null mice lack endogenous *Mtvs*.

BALB/cJ is highly susceptible to exogenous MMTV-induced mammary tumorigenesis. Milk-borne infection by MMTV(C3H) culminates with a 100% mammary tumor incidence, and a similar 90% mammary tumor incidence is observed in adult BALB/cJ females inoculated intraperitoneally with a rat fibroblast cell line stably expressing an infectious MMTV proviral clone [316] (Table 3.3). Adult BALB/cJ mice also demonstrate a 50% incidence of TBLV-induced lymphomas (Table 3.3) following intraperitoneal inoculation of Jurkat T cells stably expressing the molecular clone, HYB-TBLV [317].

BALB/cJ mice harbor three endogenous *Mtvs* - *Mtv6*, *Mtv8* and *Mtv9* on chromosomes 16, 6 and 12, respectively [65, 372]. Endogenous *Mtvs* have been demonstrated to provide resistance to infection by exogenous strains of MMTV bearing Sags of similar specificity [157, 184]. Such endogenous proviruses also serve as substrates for retroviral recombination [159, 161, 162] and, hence, may also

Table 3.3. BALB/cJ mice are highly susceptible to MMTV(C3H) and TBLV-induced tumorigenesis.

Tumor type	Mode of infection					
	Foster nursing on MMTV+ C3H/HeN mice		Intraperitoneal inoculation			
			XC/HYB-MTV		Jurkat/HYB-TBLV	
	Incidence	Average latency (months)	Incidence	Average latency (months)	Incidence	Average latency (months)
Mammary tumor	100% (6/6) ^a	5.8 ± 0.4 ^b	90% (9/10) ^a	8.9 ± 2.2 ^b	–	–
Thymic lymphoma	–	–	–	–	50% (9/18) ^c	5.7 ± 1.5 ^b

^a (Number of female mice with mammary tumors / total number of infected females). Mice that died before 12 months of age were excluded from the study.

^b Mean (± standard deviation).

^c (Number of mice with thymic lymphomas / total number of infected male and female mice).

influence infection by exogenous MMTVs of disparate Sag reactivity. Therefore, an endogenous *Mtv*-free, BALB/cJ congenic mouse strain, designated as BALB/*Mtv*-null (H-2^d), was derived for studying clonal exogenous MMTVs. This strain was selected from a cross between BALB/cJ mice (H-2^d) and the recently inbred strain PERA/Ei (H-2^k) that lacks endogenous *Mtvs* [446], followed by 10 backcrosses to the BALB/cJ strain. Thus, BALB/*Mtv*-null are congenic to BALB/cJ mice.

The absence of endogenous *Mtvs* in BALB/*Mtv*-null mice was verified by PCR analysis of mouse genomic DNA using endogenous *Mtv*-specific primers (Figure 3.14A) as well as by Southern blotting of mouse genomic DNA using

MMTV-specific probes (data not shown). We further confirmed the lack of endogenous *Mtv* Sag-mediated T-cell deletion in BALB/*Mtv*-null mice using flow cytometry (Figure 3.14B). Significant populations of endogenous *Mtv* Sag-reactive T cells - CD4+V β 3+ (*Mtv*6 Sag-specific), CD4+V β 5+ (*Mtv*6 and *Mtv*9 Sag-specific), CD4+V β 7+ (*Mtv*8 Sag-specific) and CD4+V β 12+ (*Mtv*8 and *Mtv*9 Sag-specific) - were detected in the peripheral blood lymphocytes (PBLs) of BALB/*Mtv*-null mice, indicating the absence of the corresponding endogenous *Mtvs*. These four T-cell subsets were almost completely deleted in age-matched BALB/cJ mice that harbor all three *sag*-expressing endogenous *Mtvs*.

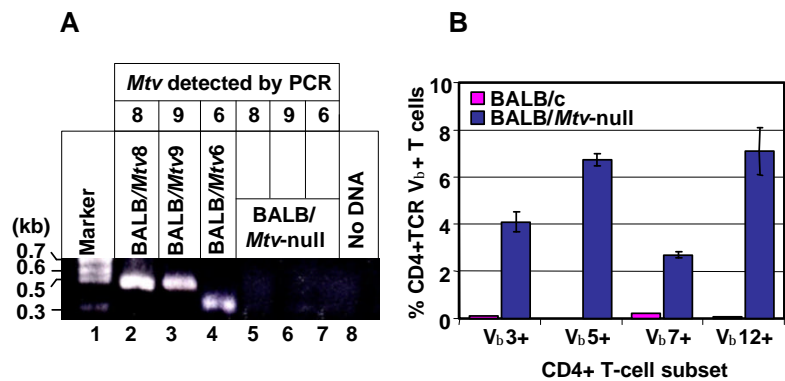


Figure 3.14. BALB/*Mtv*-null mice lack endogenous *Mtvs* present in the BALB/cJ genome.

(A) PCR analysis of mouse genomic DNA using endogenous *Mtv*-specific primers. Genomic DNA from BALB/*Mtv*6 (lane 4), BALB/*Mtv*8 (lane 2), and BALB/*Mtv*9 (lane 3) mice (harboring a single endogenous *Mtv* locus) were used as positive controls for PCR amplification of individual endogenous proviral loci. PCR with no added DNA template was the negative control (lane 8). (B) Flow cytometry analysis of endogenous *Mtv* Sag-reactive CD4+ T-cell subsets in peripheral blood lymphocytes (PBLs). PBLs from 6-month old BALB/cJ and BALB/*Mtv*-null mice were dually labeled with mouse-specific CD4-PE and mouse-specific TCR V β 3-, -5-, -7-, or -12-FITC antibodies followed by analysis on the FACSCalibur using CELLQuest software. Three individual animals of each strain were tested, and the average proportion of T-cell subsets (\pm standard deviation) is depicted.

3.2.3 BALB/*Mtv*-null strain is resistant to MMTV tumorigenesis and Sag.

BALB/*Mtv*-null and BALB/cJ mice were infected by the milk-borne route following foster-nursing on MMTV(C3H)+ C3H/HeN mice or by intraperitoneal inoculation of weanling mice with rat XC fibroblasts stably expressing HYB-MTV (XC/HYB-MTV) [316, 403]. Only a 5 to 10% incidence of mammary carcinomas was observed in the BALB/*Mtv*-null strain compared to a 90 to 100% tumor incidence in BALB/cJ mice during the 15-month observation period (Table 3.4).

Table 3.4. BALB/*Mtv*-null mice are resistant to MMTV(C3H) and TBLV-induced tumorigenesis.

Tumor type	Mode of infection					
	Foster nursing on MMTV+ C3H/HeN mice		Intraperitoneal inoculation			
			XC/HYB-MTV		Jurkat/HYB-TBLV	
	Incidence	Average latency (months)	Incidence	Average latency (months)	Incidence	Average latency (months)
Mammary tumor	10% (1/10) ^a	15	5% (1/20) ^a	9	–	–
Thymic lymphoma	–	–	–	–	0% (0/8) ^b	–

^a (Number of female mice with mammary tumors / total number of infected females). Mice that died before 12 months of age were excluded from the study.

^b (Number of mice with thymic lymphomas / total number of infected male and female mice).

Interestingly, BALB/*Mtv*-null mice inoculated with MMTV-infected rat XC cells failed to undergo MMTV(C3H) Sag-mediated peripheral deletion of CD4+V β 14+ T-cells (Figure 3.15A). In contrast, approximately 69% of Sag-

reactive CD4+V β 14+ T cells were deleted in MMTV-infected BALB/cJ mice (Figure 3.15A). BALB/*Mtv*-null mice infected neonatally with the milk-borne MMTV(C3H) demonstrated a very low-grade (18 to 20%) deletion of Sag-cognate T cells, unlike BALB/cJ mice in which approximately 59% of the CD4+V β 14+, Sag-reactive T cells were deleted within 6 months of age (Figure 3.16A and C, left panel).

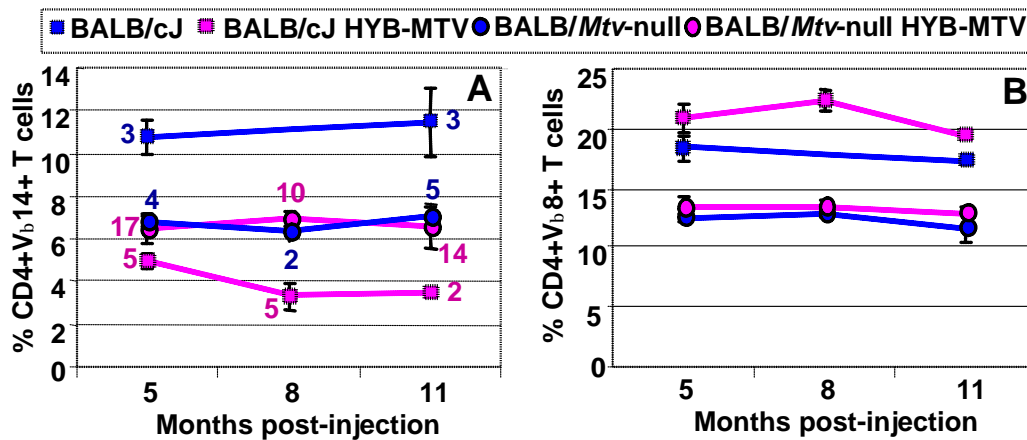


Figure 3.15. MMTV(C3H) Sag-mediated T-cell deletion is impaired in BALB/*Mtv*-null mice inoculated with XC/HYB-MTV cells.

PBLs isolated from MMTV-infected and control-uninfected mice were dually labeled with mouse-specific CD4-PE and mouse-specific V β 8, or -14-FITC antibodies followed by analysis on the FACSCalibur using CELLQuest software. The percentages of MMTV(C3H) Sag-reactive CD4+V β 14+ (A) and non-reactive CD4+V β 8+ (B) T cells among the PBLs isolated from BALB/*Mtv*-null (circles) or BALB/cJ (squares) mice inoculated with XC/HYB-MTV cells (pink) compared to uninjected mice (blue) are depicted. The number adjacent to the average %CD4+V β 14+ T-cell proportion at each time point denotes the number of mice analyzed for that time point.

The MMTV(C3H) Sag-mediated T-cell deletion in the BALB/cJ mice was specific for the Sag-reactive CD4+V β 14+ T-cell compartment while the CD4+V β 8+ T-cell subset, not recognized by MMTV(C3H) Sag, remained

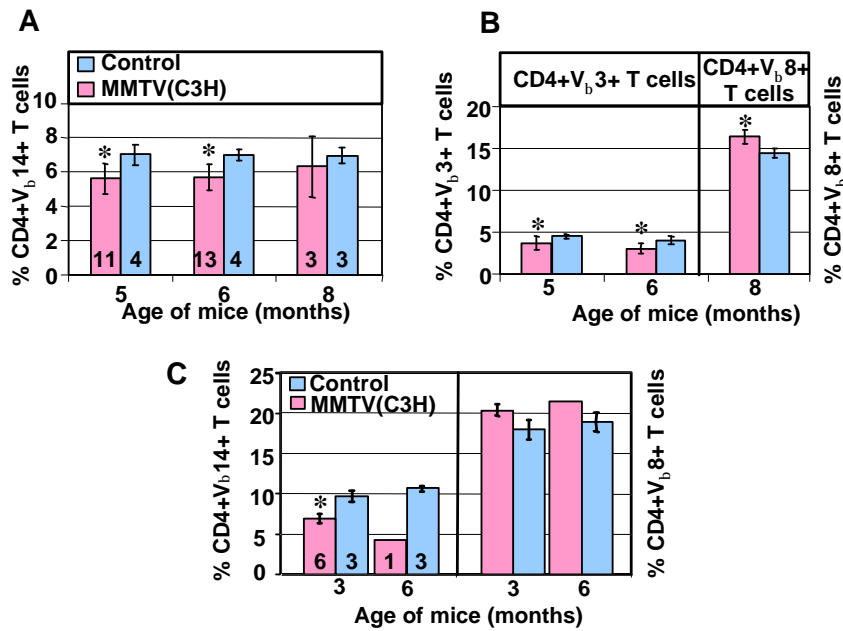


Figure 3.16. MMTV(C3H) Sag-mediated T-cell deletion is impaired in BALB/*Mtv*-null mice infected by milk-borne MMTV.

PBLs isolated from mice were dually stained with mouse-specific CD4-PE and mouse-specific V β 3, -8-, or -14-FITC antibodies followed by analysis on the FACSCalibur using CELLQuest software. Asterisks denote statistical significance determined by two-tailed Student's *t*-tests ($P < 0.05$). The percentages of MMTV(C3H) Sag-reactive (CD4+V β 14+) and non-reactive (CD4+V β 8+ or CD4+V β 3+) T cells among the PBLs isolated from infected BALB/*Mtv*-null (A, B) or BALB/cJ (C) mice (pink bars) compared to uninfected mice (blue bars) are depicted. The numbers within the bars denote the number of mice that were analyzed for that time point. Only one MMTV(C3H)-infected BALB/cJ female (C) was analyzed at 6 months since five females had been sacrificed after developing mammary tumors.

unaffected (Figure 3.15B, and Figure 3.16C, right panel). However, the CD4+V β 3+ T-cell subset, also not recognized by MMTV(C3H) Sag, underwent a low-grade deletion in BALB/*Mtv*-null mice infected with milk-borne MMTV (Figure 3.16B, left panel).

3.2.4 BALB/*Mtv*-null mice are resistant to TBLV tumorigenesis.

Sag-mediated amplification of MMTV in lymphoid cells is required for a robust infection by exogenous MMTV leading to mammary tumorigenesis [157, 161, 184]. Therefore, resistance to MMTV tumorigenesis in BALB/*Mtv*-null mice could be attributed to the lack of Sag-mediated viral amplification. To test this possibility we assessed the tumorigenicity of TBLV, a Sag-independent variant of MMTV [317], in BALB/*Mtv*-null mice. As described earlier, TBLV is a thymotropic variant of MMTV that induces thymic lymphomas in mice [26, 317]. We previously determined that TBLV-induced lymphomagenesis in BALB/cJ mice is independent of the truncated TBLV Sag [317]. Fifty percent of weanling BALB/cJ mice inoculated intraperitoneally with Jurkat cells stably expressing a hybrid proviral clone of TBLV develop lymphomas with an average latency of 5.7 months [317] (Table 3.1). In contrast, BALB/*Mtv*-null mice inoculated with the same batch of Jurkat/HYB-TBLV cells failed to develop any lymphomas within the 10-month observation period (Table 3.4). BALB/*Mtv*-null mice also were completely resistant to the *sag*-frameshift TBLV mutant, HYB-TBLV_{*sag*DFS} (data not shown) that encodes only the first 17 amino acids of Sag.

Thus, BALB/*Mtv*-null mice are resistant to both MMTV-induced mammary tumorigenesis and TBLV-induced lymphomagenesis, suggesting that the resistance phenomenon is independent of the Sag response.

3.2.5 BALB/*Mtv*-null mice develop MMTV and TBLV infection.

To determine if the resistance to TBLV and MMTV-induced tumorigenesis and impairment of MMTV Sag-mediated T-cell deletion response was due to lack of infection, we performed several RT-PCR analyses. MMTV LTR, *env* and *gag*-specific primers were used to detect MMTV transcripts in mouse organs, including the mammary gland and spleen. Despite their resistance to MMTV-induced tumorigenesis and lack of Sag-mediated T-cell deletion, BALB/*Mtv*-null mice are susceptible to MMTV infection. MMTV RNA was detected in 8 to 15-month-old BALB/*Mtv*-null mice that were infected neonatally with milk-borne MMTV(C3H) (Figure 3.17A) as well as in adult mice at 15 months post-injection with MMTV-expressing XC cells (Figure 3.17B). BALB/*Mtv*-null mice inoculated intraperitoneally with Jurkat/HYB-TBLV cells also were susceptible to infection. TBLV transcripts were detected by RT-PCR using TBLV LTR-specific primers in splenic RNA isolated from animals at 10 months post-injection (Figure 3.18A). Although BALB/*Mtv*-null mice are susceptible to MMTV and TBLV infection, viral RNA was not detected in all the BALB/*Mtv*-null mice tested. MMTV or TBLV-infected BALB/cJ mice that have not developed virus-induced tumors also do not demonstrate detectable viral RNA in their organs with a 100% frequency (data not shown), suggesting that these mice have low-level and/or sporadic infections.

MMTV-infected BALB/*Mtv*-null mice also transmitted milk-borne virus to their progeny albeit at a very low frequency (Figure 3.18B). MMTV RNA was detected by RT-PCR using *gag*-specific primers at a low frequency in the progeny

(4th to 6th litter) of BALB/*Mtv*-null mice that were foster-nursed on MMTV(C3H)+ C3H/HeN mice. However, as observed in the parental generation, the progeny mice did not demonstrate MMTV Sag-mediated T-cell deletion (data not shown).

All RT-PCR products were sequenced to confirm the authenticity of viral transcription products. Cellular *gapd* RNA was amplified by RT-PCR from all samples to ascertain RNA integrity. RT-PCRs, in which the RT had been omitted, were used to verify the absence of DNA contamination.

Thus, BALB/*Mtv*-null mice are susceptible to MMTV and TBLV infection, yet they fail to develop symptoms of infection as determined by virus-induced tumorigenesis and robust MMTV Sag-mediated T-cell deletion.

3.2.6 MMTV resistance of BALB/*Mtv*-null mice is a recessive trait.

To determine the gene(s) that are involved in the MMTV resistance trait of BALB/*Mtv*-null mice, a genetic analysis was initiated by breeding BALB/cJ x BALB/*Mtv*-null F1 mice (Figure 3.19).

Weanling F1 mice were intraperitoneally inoculated with XC/HYB-MTV cells and observed for mammary tumorigenesis and Sag-mediated peripheral T-cell deletion. Approximately 62% of the MMTV(C3H) Sag-reactive (CD4+V β 14+) T cells were deleted in the MMTV-infected F1 mice, and the kinetics of deletion was similar to that observed in BALB/cJ mice (Figure 3.20). The non-cognate CD4+V β 8+ T-cell population showed only compensatory changes.

The injected BALB/cJ x BALB/*Mtv*-null F1 mice also developed mammary tumors with incidence of 86% and an average latency of 7.3 ± 2.4 months (Table

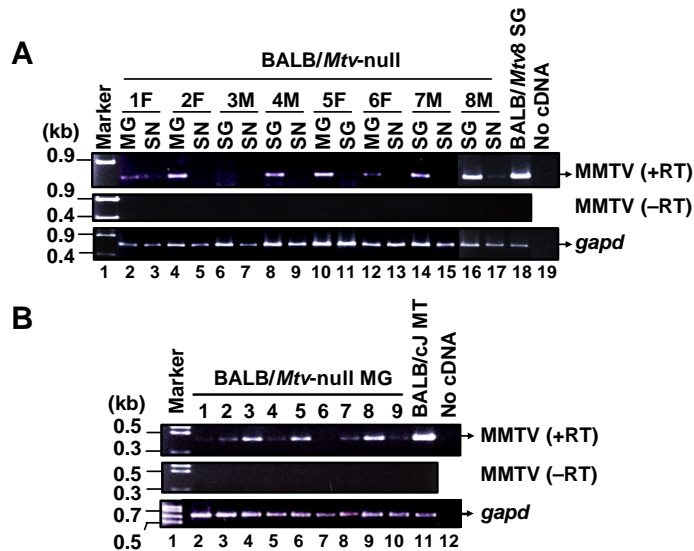


Figure 3.17. BALB/*Mtv*-null mice are susceptible to MMTV(C3H) infection.

(A) RT-PCR analysis of mammary gland (MG), spleen (SN), and salivary gland (SG) RNA from mice infected with milk-borne MMTV(C3H). RNAs from 8 to 15-month-old female (F) and male (M) BALB/*Mtv*-null mice were analyzed using LTR-specific primers C3HLTR501(+) and C3HLTR1074(-) (top panel). SG RNA from a XC/HYB-MTV injected BALB/*Mtv*8 mouse was used as a positive control. Template-minus PCR served as the negative control. Duplicate RT-minus (-RT) RT-PCRs confirmed the absence of DNA contamination (middle panel). RNA integrity was verified by PCR amplification of *gapd*-specific cDNAs (bottom panel). (B) RT-PCR analysis of MG RNA from BALB/*Mtv*-null females at 15 months post-injection with XC/HYB-MTV using *gag*-specific primers *gag*79(+) and *gag*461(-) (top panel). HYB-MTV-induced BALB/cJ mammary tumor (MT) RNA was used as a positive control. Controls for DNA contamination (middle panel) and RNA integrity (bottom panel) also are shown.

3.5). MMTV(C3H) RNA expression was detected in the mammary tumors by RT-PCR using the TBLVENV8509(+) and C3HLTR501(-) primers followed by *Cla*I RFLP analysis (Figure 3.21). These MMTV(C3H)-specific RT-PCR products give two smaller fragments after cleavage at the single *Cla*I restriction site within the amplified LTR sequence. Endogenous *Mtv*-specific RT-PCR products remain uncut since they lack this *Cla*I restriction site. *Cla*I digested RT-PCR products also were

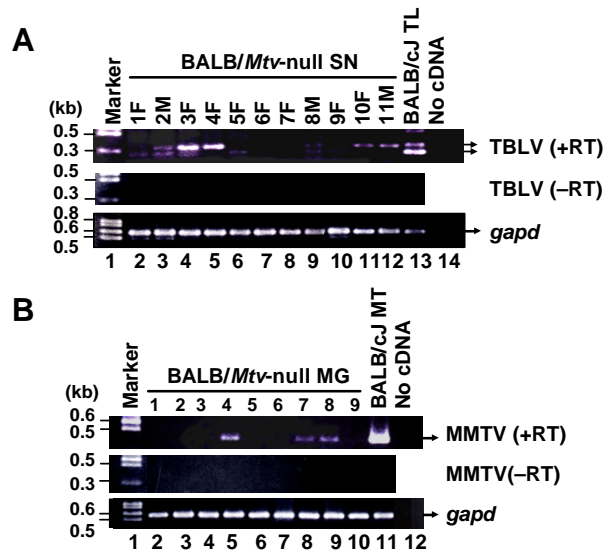


Figure 3.18. BALB/*Mtv*-null mice are susceptible to TBLV infection and transmit milk-borne MMTV(C3H) at a low frequency.

(A) RT-PCR analysis of spleen (SN) RNA from BALB/*Mtv*-null mice at 10 months post-infection with HYB-TBLV. RNA was used for RT-PCR with LTR-specific primers C3HLTR501(+) and TBLVLTR786(-) (top panel). Size variation in the TBLV-specific RT-PCR products (arrows) is due to the number of T-cell enhancer repeats in the LTR. HYB-TBLV-induced BALB/cJ thymic lymphoma (TL) RNA was used as the positive control. (B) RT-PCR analysis of mammary gland (MG) RNA from MMTV-infected BALB/*Mtv*-null mice. Reactions were performed using RNA from 4th to 6th litter female progeny of milk-borne MMTV(C3H)-infected mice and *gag*-specific primers gag79(+) and gag461(-) (top panel). HYB-MTV-induced BALB/cJ MT RNA was used as a positive control. Controls for DNA contamination (A and B, middle panels) and RNA integrity (A and B, bottom panels) also are shown.

sequenced to verify the presence of the expected viral amplification products.

Thus, resistance to MMTV tumorigenesis in BALB/*Mtv*-null mice is a recessive trait and the BALB/cJ x BALB/*Mtv*-null F1 mice are comparable to BALB/cJ mice in their susceptibility to MMTV tumorigenesis and MMTV Sag-mediated T-cell deletion.

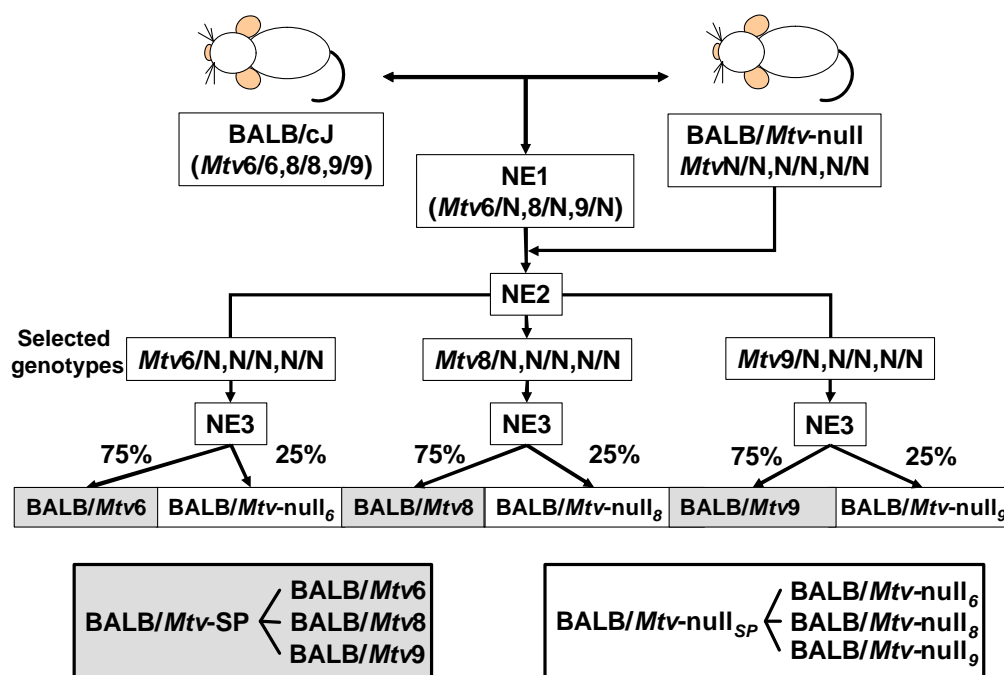


Figure 3.19. Breeding scheme of BALB/cJ x BALB/*Mtv*-null F1, BALB/*Mtv*-SP, and BALB/*Mtv*-null_{SP} mice.

F1 mice (NE1) heterozygous for all three endogenous *Mtv* loci were obtained by backcrossing the BALB/*Mtv*-null strain to the BALB/cJ strain. BALB/cJ congenic strains (NE2) heterozygous for a single endogenous *Mtv* locus (*Mtv*6, *Mtv*8 or *Mtv*9) were selected from the F1 (NE1) x BALB/*Mtv*-null backcross. Seventy-five percent of the mice in the NE3 generation were homozygous or heterozygous for a single endogenous *Mtv* locus. These animals were designated BALB/*Mtv*6, BALB/*Mtv*8 and BALB/*Mtv*9 (collectively termed BALB/*Mtv*-SP). The remaining 25% of the mice (designated BALB/*Mtv*-null_{SP}) from the same cross were null for all three endogenous *Mtvs*.

3.2.7 *Mtv* loss correlates with MMTV resistance in BALB/*Mtv*-null mice.

The BALB/*Mtv*-null strain retained its resistance to MMTV tumorigenesis after several backcrosses to the BALB/cJ strain of mice, yet BALB/cJ x BALB/*Mtv*-null F1 mice were susceptible to MMTV-induced mammary tumors. This observation suggested that the gene(s) responsible for the resistance phenotype might be linked to one or more of the endogenous *Mtv* loci that were selected

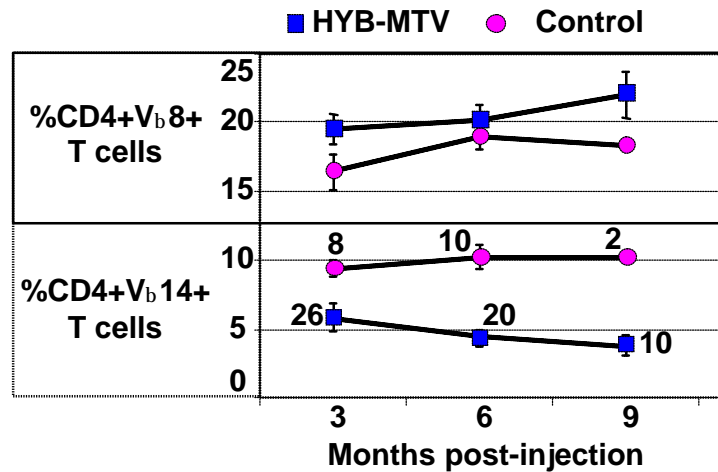


Figure 3.20. BALB/cJ x BALB/*Mtv*-null F1 mice demonstrate MMTV(C3H) Sag-mediated T-cell deletion.

PBLs isolated from XC/HYB-MTV-injected (blue squares) and control, uninfected (pink circles) BALB/cJ x BALB/*Mtv*-null F1 mice were dually stained with mouse-specific CD4-PE and V β 8- or -14-FITC antibodies followed by analysis on the FACSCalibur using CELLQuest software. The percentages of MMTV(C3H) Sag-reactive (CD4+V β 14+) and non-reactive (CD4+V β 8+) T cells among the PBLs is depicted. The number adjacent to the average %CD4+V β 14+ T-cell proportion at each time point denotes the number of mice analyzed for that time point.

against during the derivation of the BALB/*Mtv*-null mice. To determine whether the MMTV resistance trait of BALB/*Mtv*-null mice co-segregates with any of the endogenous *Mtv* loci in BALB/cJ mice, BALB/cJ congenic strains carrying a single endogenous *Mtv* locus were derived. BALB/*Mtv*6, BALB/*Mtv*8, and BALB/*Mtv*9 mice (collectively referred to as BALB/*Mtv*-SP) were derived from a BALB/cJ x BALB/*Mtv*-null cross followed by backcrossing to the BALB/*Mtv*-null strain (Figure 3.19). The same crosses also yielded siblings of BALB/*Mtv*-SP mice that lacked all three endogenous *Mtvs*. These endogenous *Mtv*-free mice, designated as BALB/*Mtv*-null_{SP}, have the same genetic background as the BALB/*Mtv*-SP mice. The presence or absence of endogenous *Mtvs* in these strains was verified

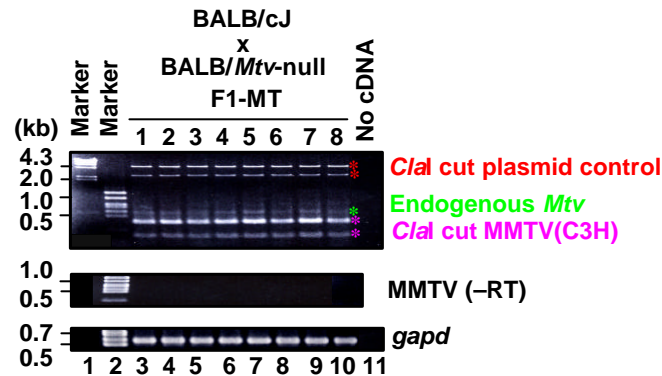


Figure 3.21. Mammary tumors induced in the XC/HYB-MTV-injected BALB/cJ x BALB/*Mtv*-null F1 mice express MMTV(C3H) transcripts.

RT-PCR analysis of RNA from MMTV-induced mammary tumors (MT) from BALB/cJ x BALB/*Mtv*-null F1 (lanes 3-10) females was followed by digestion with *Cla*I. Endogenous and exogenous MMTV-specific cDNAs were PCR amplified using MMTV *env* and LTR-specific primers TBLVENV8509(+) and C3HLTR501(-). Amplification of the endogenous *Mtv*-specific cDNAs served as an internal positive control for the PCR. PCR products were digested and used for agarose gel electrophoresis. An internal plasmid control for *Cla*I-mediated restriction digestion was included in each reaction. The results are shown in the top panel, and the positions of fragments generated by *Cla*I digestion of the internal plasmid control are marked (red asterisks). Endogenous *Mtv*-specific RT-PCR products that remain undigested by *Cla*I (green asterisk), and the two fragments generated by *Cla*I digestion of MMTV(C3H)-specific RT-PCR products (pink asterisk) are also indicated. Controls for RNA integrity (bottom panel) and lack of DNA contamination (middle panel) also are shown.

by PCR analysis of genomic DNA (Figure 3.14A) and by flow cytometric analysis of endogenous *Mtv* Sag-reactive T-cell subsets in the peripheral blood lymphocytes (data not shown).

Weanling BALB/*Mtv*-SP and BALB/*Mtv*-null_{SP} mice were inoculated intraperitoneally with XC/HYB-MTV cells and observed for MMTV(C3H) Sag-mediated T-cell deletion and mammary tumorigenesis. Interestingly, all three BALB/*Mtv*-SP strains - BALB/*Mtv*6, BALB/*Mtv*8, and BALB/*Mtv*9 - demonstrated

equivalent MMTV(C3H) Sag-mediated deletion of peripheral CD4+V β 14+ T cells with similar kinetics (Figure 3.22A). Approximately 38% of the CD4+V β 14+ T cells were deleted within 9 months post-infection in BALB/*Mtv*6 mice, whereas 48% and 45% of the CD4+V β 14+ T cells were deleted in BALB/*Mtv*8 and BALB/*Mtv*9 mice, respectively. However, the BALB/*Mtv*-null_{SP} siblings lacked Sag-mediated T-cell deletion, a phenotype similar to the original BALB/*Mtv*-null mice. The CD4+V β 8+ T-cell subset, not recognized by MMTV(C3H) Sag, remained unaffected in all mouse strains under investigation (Figure 3.22B), validating the specificity of the Sag-mediated T-cell deletion response observed in BALB/*Mtv*-SP mice.

BALB/*Mtv*-SP mice were also susceptible to MMTV induced mammary tumorigenesis. The incidence of mammary carcinomas was highest within the BALB/*Mtv*8 strain since 75% of the females developed tumors with an average latency of 9.1 ± 3.6 months (Table 3.5). Thirty eight percent of the BALB/*Mtv*6 females and 67% of the BALB/*Mtv*9 females developed mammary tumors with an average latency of 12.8 ± 1.8 and 11.5 ± 3.5 months, respectively. Two out of the 9 BALB/*Mtv*-null_{SP} females (22%) also developed mammary tumors at 14.5 ± 0.7 months post-infection.

Statistical analysis revealed that the BALB/*Mtv*6 and BALB/*Mtv*8 strains of mice had a significantly higher susceptibility to MMTV-induced tumorigenesis compared to the BALB/*Mtv*-null strain (Figure 3.23). BALB/*Mtv*9 mice also demonstrated an increased susceptibility to MMTV-induced tumorigenesis compared to the BALB/*Mtv*-null strain; however, this difference was only

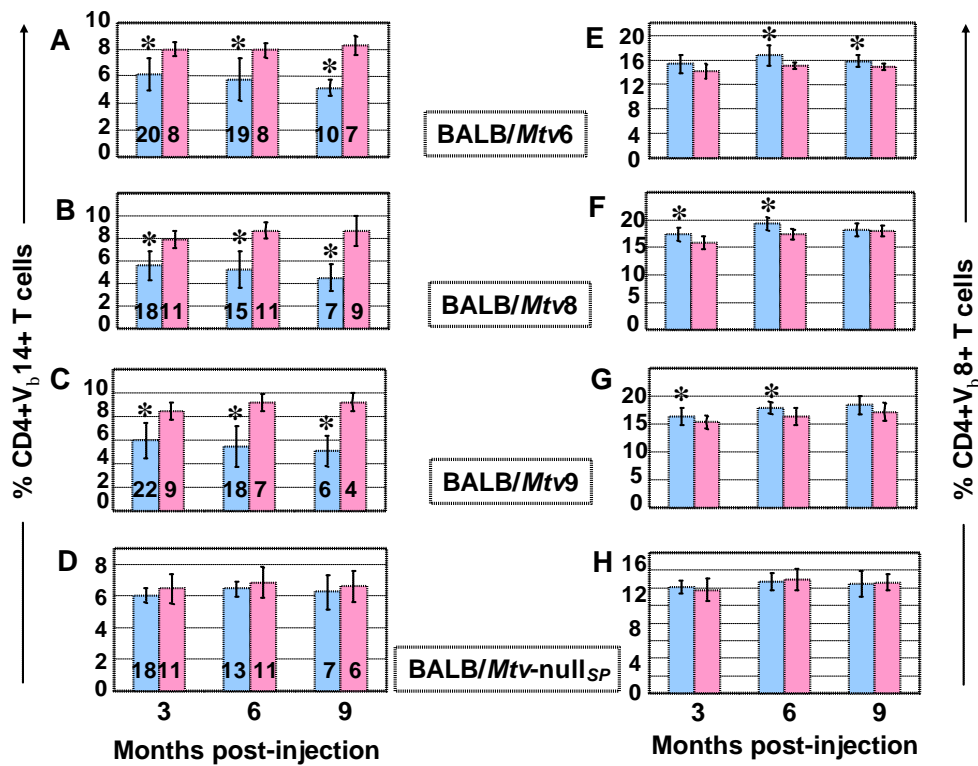


Figure 3.22. BALB/*Mtv*-SP mice demonstrate MMTV(C3H) Sag-mediated T-cell deletion.

The percentages of Sag-reactive (A-D) CD4+V β 14+ and non-reactive (E-H) CD4+V β 8+ T cells among the PBLs isolated from MMTV-infected (blue) and control uninfected (pink) BALB/*Mtv6* (panels A and E), BALB/*Mtv8* (panels B and F), BALB/*Mtv9* (panels C and G), and BALB/*Mtv-null_{SP}* (panels D and H) mice are depicted. Asterisks indicate statistical significance determined by two-tailed Student's *t*-test ($P < 0.05$). The numbers within each bar denote the number of mice analyzed for that time point.

marginally significant due to an unusually high attrition of BALB/*Mtv9* females owing to reproductive complications. MMTV(C3H) RNA expression was detected in the mammary tumors by RT-PCR using TBLVENV8509(+) and C3HLTR501(-) primers. Products also were verified by *Cla*I RFLP analysis followed by sequencing of the *Cla*I-digested RT-PCR products (Figure 3.24).

Therefore, the phenomenon of susceptibility to MMTV tumorigenesis and

Table 3.5. BALB/cJ x BALB/*Mtv*-null F1 and BALB/*Mtv*-SP mice are susceptible to MMTV-induced tumorigenesis.

Strain ^a	Mammary tumor incidence ^b	Average latency (months)
BALB/cJ x BALB/ <i>Mtv</i> -null F1	86% (12/14)	7.3 ± 2.4 ^c
BALB/ <i>Mtv</i> 6	38% (5/13)	12.8 ± 1.8
BALB/ <i>Mtv</i> 8	75% (6/8)	9.1 ± 3.6
BALB/ <i>Mtv</i> 9	67% (2/3)	11.5 ± 3.5
BALB/ <i>Mtv</i> -null _{SP}	22% (2/9)	14.5 ± 0.7

^a Weanling mice were inoculated intraperitoneally with 2×10^7 XC/HYB-MTV cells and observed for 15 months.

^b (Number of female mice with mammary tumors / total number of infected females). Mice that died before 12 months of age, generally due to birthing problems, were excluded from the study.

^c Mean (± standard deviation).

Sag-mediated T-cell deletion appears to co-segregate with all three endogenous *Mtv* loci in BALB/cJ mice. This observation suggests that the resistance of BALB/*Mtv*-null mice to MMTV infection correlates with their lack of endogenous *Mtvs*, rather than linkage of three recessive genes with each provirus on three separate chromosomes.

3.2.8 BALB/*Mtv*-null mice lack MMTV-neutralizing antibodies.

Since the presence of any one of the endogenous *Mtvs* of BALB/cJ mice was sufficient to render the BALB/*Mtv*-null mice susceptible to MMTV tumorigenesis, we hypothesized that BALB/cJ, but not BALB/*Mtv*-null mice, have

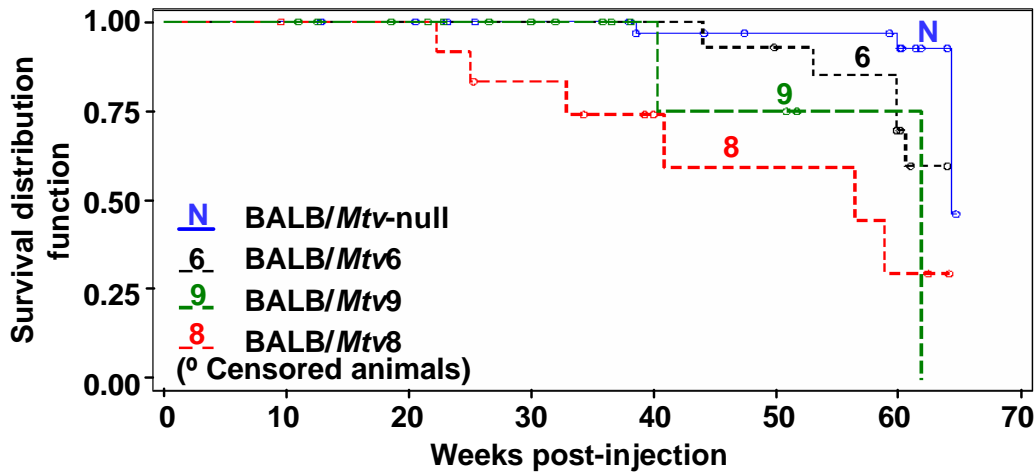


Figure 3.23. Statistical analysis of MMTV-induced mammary tumorigenesis in the BALB/*Mtv*-SP mice.

Kaplan-Meier survival analysis of the susceptibility of BALB/*Mtv*6, BALB/*Mtv*8, BALB/*Mtv*9, and BALB/*Mtv*-null mice to MMTV tumorigenesis. The LIFETEST procedure in SAS 8.2 was used to compute nonparametric estimates of the survivor function by the product-limit method (also called the Kaplan-Meier method). The homogeneity of survival functions across groups was tested using the log-rank test and the Wilcoxon test. The P values for the pair-wise comparisons were as follows - BALB/*Mtv*-null versus BALB/*Mtv*6: $P_{Log-rank} = 0.0264$; $P_{Wilcoxon} = 0.0445$; BALB/*Mtv*-null versus BALB/*Mtv*8: $P_{Log-rank} < 0.0001$; $P_{Wilcoxon} < 0.0001$; BALB/*Mtv*-null versus BALB/*Mtv*9: $P_{Log-rank} = 0.0094$; $P_{Wilcoxon} = 0.0674$.

immune tolerance to MMTV. Therefore, following MMTV infection, BALB/*Mtv*-null mice may develop a strong MMTV-specific neutralizing antibody response that would curtail a robust infection required for tumorigenesis. To assess the anti-MMTV humoral response, BALB/cJ and BALB/*Mtv*-null mice were foster-nursed on MMTV+ C3H/HeN mice followed by analysis of MMTV-specific antibodies in sera obtained weekly (Figure 3.25A). BALB/cJ mice sporadically developed virus-specific antibodies during the first 3 to 5 weeks of age as reported previously [350]. This humoral response was transient since MMTV-specific antibodies were

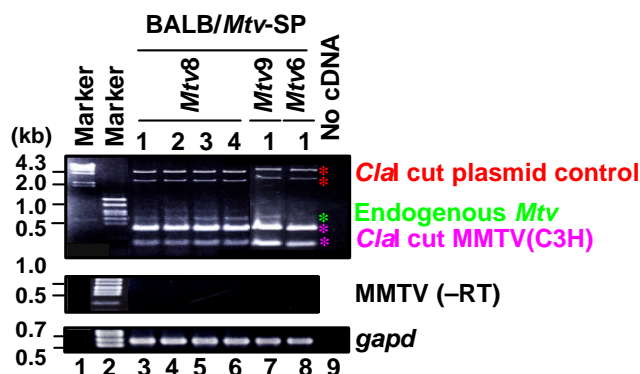


Figure 3.24. Mammary tumors induced in the XC/HYB-MTV-injected BALB/*Mtv*-SP mice express MMTV(C3H) transcripts.

RT-PCR was performed using RNA from MMTV-induced mammary tumors from four BALB/*Mtv*8 (lanes 3-6), 1 BALB/*Mtv*9 (lane 7), and 1 BALB/*Mtv*6 (lane 8) females. Endogenous and exogenous MMTV-specific cDNAs were amplified by PCR using MMTV *env* and LTR-specific primers TBLVENV8509(+) and C3HLTR501(-). Amplification of the endogenous *Mtv*-specific cDNAs served as an internal positive control for the PCR. PCR products were then digested with *Cla*I followed by agarose gel electrophoresis. An internal plasmid control for *Cla*I-mediated restriction digestion was included in each reaction. The results are shown in the top panel, and the positions of fragments generated by *Cla*I digestion of the internal plasmid control are marked (red asterisks). Endogenous *Mtv*-specific RT-PCR products that remain undigested by *Cla*I (green asterisk), and the two fragments generated by *Cla*I digestion of MMTV(C3H)-specific RT-PCR products (pink asterisks) are also indicated. Controls for RNA integrity (bottom panel) and lack of DNA contamination (middle panel) also are shown.

undetectable in older (6 to 9 week-old) mice. Serum antibodies against MMTV were also detected in about 30% of the BALB/*Mtv*-null mice infected by milk-borne MMTV, and some individuals demonstrated a higher antibody titer than that obtained in infected BALB/cJ mice. The anti-MMTV response was also maintained for a longer duration in these BALB/*Mtv*-null mice compared to the BALB/cJ mice. The serum antibodies from BALB/*Mtv*-null mice were specific for MMTV capsid as determined by Western blotting against purified MMTV virions (Figure 3.25B).

BALB/*Mtv*-null serum antibodies did not demonstrate any reactivity with MMTV Env proteins, suggesting their inability to function in virus neutralization.

Thus, the resistance of BALB/*Mtv*-null mice towards MMTV tumorigenesis is not due to the presence of virus-specific neutralizing antibodies.

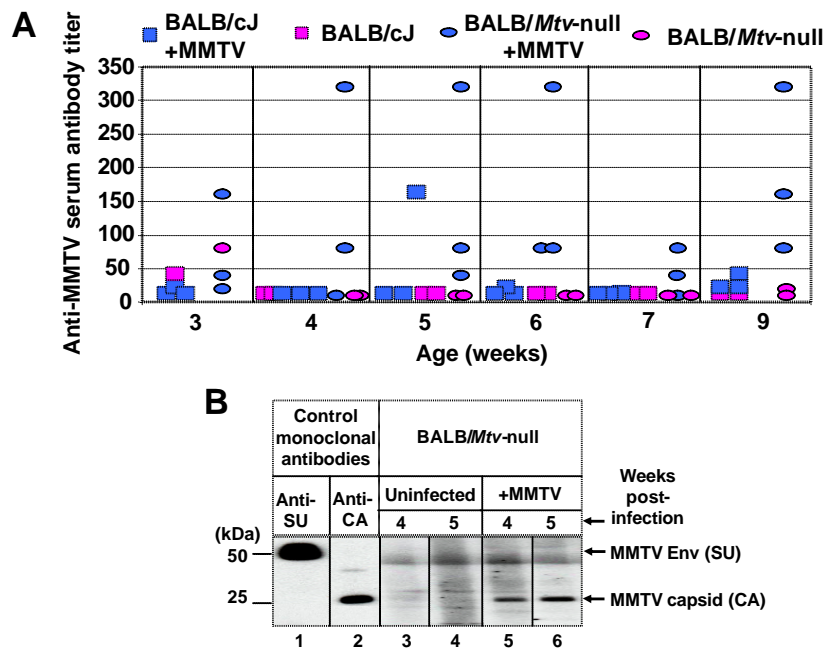


Figure 3.25. BALB/*Mtv*-null mice infected by milk-borne MMTV develop MMTV CA-specific serum antibodies.

(A) Sera from milk-borne MMTV(C3H)-infected (blue) or age-matched uninfected (pink) BALB/cJ (squares) and BALB/*Mtv*-null (circles) mice were analyzed weekly by ELISA to detect MMTV virion-specific antibodies. Each symbol represents an individual mouse. The serum antibody titer was calculated as the reciprocal of the highest serum dilution that reacted specifically with MMTV virions. (B) Western blot against MMTV virion proteins using a 1:20 dilution of sera (titer = 320) from MMTV(C3H)-infected BALB/*Mtv*-null mice at 4 to 5 weeks of age. MMTV CA or SU-specific monoclonal antibodies were used as positive controls. Sera from two age-matched uninfected BALB/*Mtv*-null mice were used as negative controls.

3.2.9 BALB/*Mtv*-null mice reject MMTV-expressing BALB/cJ B-cells.

The resistance of BALB/*Mtv*-null mice towards MMTV tumorigenesis also may result from a cell-mediated immune response against virus-infected cells. To evaluate this hypothesis, weanling BALB/*Mtv*-null and BALB/cJ mice were inoculated with 5×10^6 A20 (BALB/c B-cell lymphoma line) cells [221]. A second set of mice were inoculated with 5×10^6 A20 cells stably expressing HYB-MTV (A20/HYB-MTV) (Figure 3.26). The proliferation of A20 cells resulted in solid, peritoneal tumors in 25% of the inoculated BALB/cJ mice with an average latency of 7.5 ± 1.5 weeks (Table 3.6). BALB/*Mtv*-null mice had a similar tolerance towards A20 cells since 29% of these mice developed peritoneal A20 tumors within 6.8 ± 2.3 weeks. Thirty eight percent of the BALB/cJ mice also developed large, peritoneal outgrowths within 5.2 ± 0.5 weeks of inoculation with MMTV-expressing A20 cells (Table 3.6). In contrast, the BALB/*Mtv*-null mice completely rejected the same dosage of A20/HYB-MTV cells and failed to develop any tumors.

This experiment provides preliminary evidence that the resistance towards MMTV tumorigenesis in the BALB/*Mtv*-null strain might be influenced by the cell-mediated cytotoxic immune response of these mice towards MMTV-infected cells.

3.2.10 BALB/*Mtv*-null mice are resistant to *Vibrio cholerae*.

Since the resistance of BALB/*Mtv*-null mice towards MMTV and TBLV-induced tumorigenesis might be an immunological phenomenon, it is possible that this mouse strain has an altered response to other pathogens. The susceptibilities of BALB/cJ and BALB/*Mtv*-null mice towards *V. cholerae* strain 0395, which

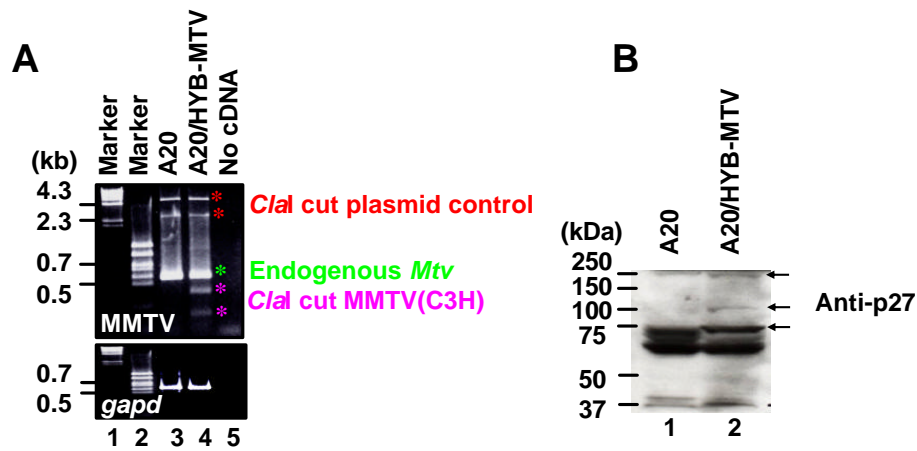


Figure 3.26. Characterization of A20/HYB-MTV cells.

(A) HYB-MTV RNA expression in A20/HYB-MTV cells was verified by RT-PCR and *Cla*I RFLP analysis. MMTV-specific cDNAs were PCR-amplified using *env* and LTR-specific primers TBLVENV8509(+) and C3HLTR501(-). Amplification of the endogenous *Mtv*-specific cDNAs served as an internal positive control. PCR products were digested with *Cla*I followed by agarose gel electrophoresis (top panel). An internal plasmid control for *Cla*I-mediated restriction digestion was included in each reaction (red asterisks). Endogenous *Mtv*-specific RT-PCR products that remain undigested by *Cla*I (green asterisk) and the two fragments generated by *Cla*I digestion of HYB-MTV-specific RT-PCR products (pink asterisks) are also indicated. RT-PCR amplification of *gapd* mRNA served as a control for RNA integrity and quantitation (B) Western blot analysis of MMTV Gag expression in A20/HYB-MTV cells. Gag precursors (indicated by arrows) were detected in 100 μ g of whole cell lysates (WCL) with MMTV CA-specific monoclonal antibody [350]. WCL from untransfected A20 cells was used as a control to determine the level of Gag expression from endogenous *Mtvs*.

expresses cholera toxin, were compared by intragastric inoculation of 5 to 6-day-old pups with 50 μ l of bacterial suspensions containing 10^5 , 10^6 or 10^7 CFU of mid-log phase bacteria. The average LD_{50} of *V. cholerae* 0395 in BALB/cJ mice was calculated to be 1.6×10^5 CFU (Table 3.8). In contrast, the average LD_{50} of *V. cholerae* in BALB/*Mtv*-null mice was 10-fold higher (2.3×10^6 CFU) (Table 3.7).

To evaluate whether the increased resistance to *V. cholerae* in the

Table 3.6. BALB/*Mtv*-null mice reject BALB/c B-cell lymphoma stably expressing HYB-MTV.

Mouse strain	A20		A20/HYB-MTV	
	Tumor incidence ^a	Average latency (weeks) ^b	Tumor incidence ^a	Average latency (weeks) ^b
BALB/cJ	25% (3/12)	7.5 ± 1.5	38% (5/13)	5.2 ± 0.5
BALB/ <i>Mtv</i> -null	29% (4/14)	6.8 ± 2.3	(0/14)	—

^a A20 and A20/HYB-MTV cells formed solid tumors in the peritoneum. The incidence is calculated as a percentage, and the number of mice with tumors versus the number of inoculated mice is shown within brackets.

^b Mean (± standard deviation).

BALB/*Mtv*-null strain was linked to the absence of endogenous *Mtvs*, the susceptibilities of the BALB/*Mtv*6, BALB/*Mtv*8 and BALB/*Mtv*9 strains of mice to *V. cholerae* infection were determined. The average LD₅₀ of *V. cholerae* in these three strains was 2.25×10^5 , $<1.2 \times 10^5$, and 2×10^5 , respectively (Tables 3.9, 3.10, and 3.11) indicating that the susceptibilities of BALB/*Mtv*6, BALB/*Mtv*8, and BALB/*Mtv*9 mice to *V. cholerae* infection were comparable to that of the BALB/cJ strain. The results were analyzed by single-factor ANOVA followed by Tukey's post-hoc test using SPSS 12.0. A significant difference ($P < 0.05$) was observed between the average LD₅₀ of BALB/*Mtv*-null mice compared to the BALB/cJ strain, while the BALB/*Mtv*-SP mice were statistically similar to the BALB/cJ strain in their susceptibilities to *V. cholerae*. Thus, susceptibility to *V. cholerae* co-segregates with endogenous *Mtv* loci located on separate chromosomes. These data indicate

Table 3.7. LD₅₀ of *Vibrio cholerae* 0395 in BALB/*Mtv*-null mice.

Experiment	Dose	Dead/Live pups	LD ₅₀
1	9×10^7	4/0	1.3×10^6
	8×10^6	4/0	
	4×10^5	0/4	
2	8.5×10^6	2/1	1.6×10^6
	9×10^5	2/2	
	6.5×10^4	0/4	
3	7.5×10^6	2/1	5×10^6
	9×10^5	0/2	
	9×10^4	0/3	
4	1.5×10^7	3/0	1.5×10^6
	5×10^5	0/4	
	2×10^5	0/4	
Average LD ₅₀			2.3×10^6

that the lower mortality rate following *V. cholerae* infection in the BALB/*Mtv*-null strain is also due to the absence of endogenous *Mtvs*.

These results elucidate a generic resistance mechanism in the BALB/*Mtv*-null mice that provides a heightened resistance to disparate pathogens, MMTV and *V. cholerae*. The resistance to both pathogens is linked to the absence of endogenous *Mtvs* in the BALB/*Mtv*-null mice.

Table 3.8. LD₅₀ of *Vibrio cholerae* 0395 in BALB/cJ mice.

Experiment	Dose	Dead/Live pups	LD ₅₀
1	2×10^7	3/1	2.2×10^5
	6×10^5	3/1	
	5×10^4	1/2	
2	3.5×10^7	4/0	1×10^5
	4×10^6	4/0	
	1×10^5	2/2	
3	3×10^7	2/0	$< 8.5 \times 10^4$
	4.5×10^6	3/0	
	8.5×10^4	2/1	
Average LD ₅₀			1.6×10^5

Table 3.9. LD₅₀ of *Vibrio cholerae* 0395 in BALB/Mtv6 mice.

Experiment	Dose	Dead/Live pups	LD ₅₀
1	7.5×10^6	3/0	2×10^5
	9×10^5	3/1	
	9×10^4	1/1	
2	8.5×10^6	2/1	4×10^5
	9×10^5	3/1	
	6.5×10^4	0/3	
3	4×10^7	3/0	2×10^5
	3.5×10^6	4/0	
	1×10^5	1/3	
4	2×10^6	2/0	1×10^5
	1×10^5	3/0	
	3.5×10^4	0/3	
Average LD ₅₀			2.25×10^5

Table 3.10. LD₅₀ of *Vibrio cholerae* 0395 in BALB/Mtv8 mice.

Experiment	Dose	Dead/Live pups	LD ₅₀
1	2×10^7	4/0	8×10^4
	1×10^6	4/1	
	5×10^4	2/2	
2	2×10^7	3/0	$< 1.2 \times 10^5$
	2.7×10^6	4/1	
	1.2×10^5	2/0	
3	2.4×10^7	3/0	$< 1.6 \times 10^5$
	7.5×10^6	2/0	
	1.6×10^5	2/1	
Average LD ₅₀			$< 1.2 \times 10^5$

Table 3.11. LD₅₀ of *Vibrio cholerae* 0395 in BALB/Mtv9 mice.

Experiment	Dose	Dead/Live pups	LD ₅₀
1	1.25×10^7	4/0	8.4×10^4
	9×10^5	4/0	
	4×10^4	1/3	
2	1.5×10^7	3/0	2.2×10^5
	1.5×10^6	3/0	
	7.5×10^4	0/3	
3	4.3×10^7	3/0	2×10^5
	2.3×10^6	5/0	
	9×10^4	1/3	
4	1×10^7	2/0	3×10^5
	1×10^6	3/0	
	1×10^5	0/3	
Average LD ₅₀			2×10^5

Discussion

4.1 Conversion of MMTV to a Lymphomagenic Virus

4.1.1 A novel TBLV tumorigenicity assay

A molecular clone of TBLV (HYB-TBLV) was constructed to study the life cycle and tumorigenicity of a previously heterogeneous virus. HYB-TBLV was engineered by substituting the *Cla*I to *Sst*I region of the TBLV LTR into the corresponding region of the 3' LTR of HYB-MTV [403]. Adult BALB/cJ mice inoculated intraperitoneally with Jurkat T cells stably expressing HYB-TBLV consistently developed a 50% incidence of T-cell lymphomas with a latency of 5.7 ± 1.5 months. A similar change in the tumor target tissue, from mammary gland to thymus, was reported for recombinant viruses obtained by substitution of the 3' LTR of HYB-MTV with the entire LTR of thymotropic MMTV variants MA and DL-8 [475]. Lymphomagenicity of HYB-TBLV allowed the definition of a smaller (438 bp) region (encompassing the LTR deletion and substitution) of the thymotropic LTR that is sufficient for altering the tumorigenicity of the recombinant provirus.

Rat XC fibroblasts [418] have been widely used for introducing the MMTV molecular clone, HYB-MTV, into mice [403]. However, XC-cell transfectants of HYB-TBLV were highly inconsistent in transmitting infection resulting in tumorigenesis in mice [Mustafa, F. *et. al.*, unpublished observations). This inefficiency might be due to the low infectious titer of virus produced by XC cells. Additionally, XC cells do not release extracellular virions, which might have a significant influence on the infection efficiency. Jurkat T cells were selected as carriers of HYB-TBLV with the assumption that the T-cell-specific enhancer in the TBLV LTR would allow elevated viral transcription in T cells, leading to the production of higher viral titers. Jurkat T cells also release extracellular virions [317] that might facilitate a greater efficiency of infection and hence tumorigenesis. Inherent properties of the cell lines might also influence the infectiousness of the stable transfectants. The different nature of the immune response and cell-cell interactions induced by XC cells and Jurkat T cells may have a significant influence on the ability of these cells to transfer the virus to host cells. It has been suggested that dissemination of MMTV infection is mediated mainly by cell-cell contact rather than free viral particles [379]. Neither XC nor Jurkat cells induce tumors in mice due to the host immune reaction against the xenogeneic cell lines [410].

Previous studies on TBLV were performed by intrathymic inoculation of newborn pups with heterogeneous virus-containing cultured lymphoma-cell supernatants [21, 22, 23, 375]. Thymic lymphomas developed in 90% of these animals within 42 to 60 days of inoculation [21, 22, 23, 309, 375]. Several factors might have contributed to the lower incidence and longer latency of HYB-

TBLV-induced lymphomas. First, since the thymus starts to regress in adult mice [148, 193], fewer proliferating thymocytes would be available for infection and random insertional mutagenesis by TBLV [59, 358]. The ages of the thymic and bone marrow microenvironments were demonstrated to influence the induction of radiation-induced thymic lymphomas in mice [437]. The tumor incidence was much higher in younger microenvironments as compared to tissues from older mice, perhaps due to secreted growth factors. Second, the route of infection might influence the efficiency of tumorigenesis. Lymphoma cell supernatants were introduced into newborn mice intrathymically, thus directly inoculating TBLV into the target for oncogenesis, whereas HYB-TBLV-expressing Jurkat cells were introduced into the mouse peritoneum, requiring virus transmission by an unknown mechanism to thymocytes or their progenitors. Third, the infectious viral titer introduced into mice by injecting 485-10 lymphoma cell supernatants might have been much higher compared to the infectious titer obtained from Jurkat cells expressing HYB-TBLV. Fourth, it is possible that the culture supernatants from 485-10 cells contained a mixture of TBLV strains, which contributed to the transforming activity compared to the clonal HYB-TBLV produced by Jurkat T cells.

Thymic lymphomas induced by HYB-TBLV were phenotypically similar to the lymphomas induced by uncloned virus-containing culture supernatants from 485-10 lymphoma cells [309, 310]. In each case, the lymphomas were oligoclonal as determined by Southern blot analysis of TCR β and γ chain rearrangements. Tumor cell populations were Thy1.2+ and heterogeneous for CD4 and CD8 surface markers. Most tumors had an abnormal proportion of

CD4/CD8 double-positive, double-negative and single-positive cells compared to the ratio of these compartments in a normal thymus. Oligoclonal tumors might arise by proliferation and differentiation, involving TCR gene rearrangements and co-receptor expression, of a single transformed pluripotent precursor cell [50]. Abnormal proportions of thymocyte compartments could also result from abnormal differentiation of transformed progenitor cells. Oligoclonality might also arise from transformation of multiple thymocytes that have undergone independent TCR gene rearrangements and selection for co-receptor usage [50]. However, the finding that TBLV-induced tumors have multiple integrations near *c-myc* and *Rorγ* [59, 60] and the detection of distinct TCR rearrangements suggest that multiple cells are transformed by TBLV followed by differential growth selection of these cells.

Thus, the T-cell lymphomas induced by the TBLV hybrid proviral clone in adult mice are comparable to those induced by inoculation of newborn mice with the culture supernatant from 485-10 lymphoma cells. Development of a cloned TBLV provirus-based tumorigenicity assay provides a valuable tool for the molecular dissection of viral elements required for determining MMTV disease specificity.

4.1.2 Sag-independent TBLV lymphomagenesis

A *sag*-frameshift provirus (HYB-TBLV_{*sag*}DFS), encoding only the first 17 amino acids of Sag, was engineered to determine the role of the truncated Sag in TBLV-induced lymphomagenesis. The incidence (60%) and latency (4.8 ± 0.6 months) of lymphoma induction by HYB-TBLV_{*sag*}DFS was not significantly different from the wild-type HYB-TBLV. The frameshift virus induced lymphomas

that were oligoclonal as determined by Southern blotting analysis of TCR β and γ chain rearrangements. These tumors also were not significantly different from HYB-TBLV-induced lymphomas with respect to the cell surface markers: CD4, CD8 and Thy1.2. Thus, truncated Sag is not required for TBLV-induced tumorigenesis in BALB/cJ mice and might be specific for the intraperitoneal route of infection. Alternatively, the Sag proteins expressed from the endogenous *Mtvs* in the BALB/cJ mice may complement the *sag*-null TBLV provirus. However, it is likely that the truncated Sag has no role in TBLV-induced tumorigenesis and that *cis*-acting LTR transcriptional elements are critical for TBLV tumorigenicity.

4.1.3 Similarity of MMTV NRE with insulators

T-cell enhancer position with respect to the NRE within the MMTV LTR influences its contribution toward transcriptional efficiency. When placed downstream of the NRE adjacent to the promoter, the enhancer gives 700-fold increased transcription from the MMTV LTR in Jurkat T cells. However, when the NRE is situated between the enhancer and the promoter, the transcriptional influence of the enhancer is greatly mitigated. Thus, the MMTV NRE, which binds CDP and SATB1, represses the TBLV enhancer in mutant MMTV LTRs in a position-dependent manner.

Binding of SATB1 and CDP to matrix attachment regions (MARs) is known to repress transcriptional enhancers by – (i) antagonizing transcriptional activators, e.g., CDP binding to flanking MAR elements interferes with Bright (B-cell regulator of IgH transcription) at the immunoglobulin heavy chain intronic enhancer

($E\mu$) [453] and (ii) modulating the chromatin structure, e.g., at $MAR\beta$ located upstream of the $TCR\beta$ enhancer ($E\beta$) [74, 220]. However, the position sensitivity of the NRE-mediated repression is reminiscent of the action of insulator elements. A defining property of insulators is their interference with the activity of silencers and enhancers when placed between the regulatory element and the promoter, whereas such regulatory elements are not inhibited in a promoter-proximal location [142]. Insulator sequences appear to function by several different mechanisms, including – insulator-insulator interactions that form chromatin loop domains; recruitment of chromatin-modifying/remodelling complexes, either directly or via general regulatory factors (GRFs), which act as landing platforms; enhancer capture involving decoy promoters; association with nuclear pore complexes; and interaction with the nuclear matrix via MAR/SAR (scaffold attachment region) domains [142, 235, 319]. Interestingly, *SATB1* has been proposed to function as a GRF at insulators [142]. Moreover, its negative regulatory activity for the MMTV promoter requires interaction with the nuclear matrix [401].

4.1.4 Increased glucocorticoid induction of MMTV by the TBLV enhancer

The MMTV LTR demonstrated a 5-fold glucocorticoid induction of its transcriptional activity in HC11 cells. The TBLV and MMTV LTRs both have six HREs due to triplication of an HRE half-site within the T-cell enhancer and removal of the two HREs located within the NREs [27, 141, 338]. However, compared to MMTV, the TBLV LTR demonstrated an enhanced glucocorticoid response that may result from absence of NRE-mediated repression of hormone-induced

transcription [483]. Also, the HREs embedded within the MMTV NREs [141] may not function effectively in the glucocorticoid induction of MMTV LTR-driven transcription. Sequences surrounding MMTV HREs are known to influence the activity of the HREs by determining the recruitment of co-factors, which destabilize GR binding to the HREs in a cell-type specific manner [203]. Furthermore, a region (between -163 to -147 bp from the transcription start site) within the MMTV HRE has been implicated in binding nuclear factors that negatively regulate the hormone responsiveness of the LTR in certain cell lines [420]. The distance between the MMTV TATA box and the HREs also influences the transcriptional activation since altering the distance beyond the optimum decreases transcriptional activation [180].

The MMTV mutant *MTVA Δ E* LTR harbors nine HREs yet its glucocorticoid responsiveness is similar to that of the TBLV LTR. This might result from inefficient GR binding to the HREs located within the NRE. The *MTV Δ StuE* LTR also harbors nine HREs, but demonstrates a significantly lower glucocorticoid induction compared to both TBLV and *MTVA Δ E* LTRs. Thus, as discussed in Section 4.1.3, the position of the NRE with respect to the enhancer influences its activity, including the glucocorticoid response of the MMTV LTR.

The NREs repress both hormone-induced and basal transcription from the MMTV LTR [483]. Thus, the *MTV Δ NRE* LTR lacking the entire NRE shows an increased basal transcription in HC11 cells. However, the TBLV LTR also lacks the NRE but does not demonstrate a significantly higher basal transcription compared to the MMTV LTR, suggesting that the T-cell enhancer might have a negative influence on the basal MMTV transcription in HC11 mammary cells. The

MTV Δ NRE LTR harbors three HREs but does not show a significantly higher induction by glucocorticoids. Since hormone receptor-binding sites in the MMTV LTR act synergistically [20] a reduction in the number of HREs in the LTR would reduce the efficiency of glucocorticoid induction.

4.1.5 Requirement of a T-cell enhancer and loss of NRE activity in lymphomagenic MMTVs

Unlike wild-type HYB-MTV, which exclusively induces mammary tumors in BALB/cJ mice, HYB-MTV Δ fE and HYB-MTV Δ StuE induced both mammary tumors and thymic lymphomas in the infected mice. The lower incidence of tumor induction by the HYB-MTV Δ StuE and HYB-MTV Δ fE viruses might be within experimental variation; however, the lower transcriptional efficiency of the MTV Δ StuE LTR compared to the TBLV LTR in T cells may result in a slower viral amplification and a decreased rate of oncogenesis. Additionally, the time required for the selection of HYB-MTV Δ StuE recombinants might also contribute towards the lower incidence of lymphomagenesis. The transcriptional strength of the MTV Δ fE LTR is comparable to that of the TBLV LTR; however, unlike TBLV, the MTV Δ fE virus induced Sag-mediated T-cell deletion. This deletion is also statistically faster than the T-cell deletion observed in HYB-MTV infected mice. The low tumor incidence of the HYB-MTV Δ fE virus might be a by-product of the rapid deletion of T cells that might serve as oncogenesis targets and also function in viral amplification and spread. Rapid Sag-mediated T-cell depletion has been correlated with a low mammary tumor incidence in adult mice infected

with MMTV(SW) [335]. The significantly faster Sag-mediated T-cell deletion in mice infected by HYB-MTVA Δ E might result from the higher viral loads generated by enhanced viral replication in T cells. However, faster kinetics of Sag-mediated T-cell depletion may be due to experimental variation.

RT-PCR and Southern blot analysis of lymphoma RNA and DNA revealed that both the T-cell enhancer and the NREs were retained in the predominant HYB-MTVA Δ E proviruses. In contrast, proviruses containing only the T-cell enhancer and lacking the NREs were selected in all the HYB-MTV Δ StuE-induced thymic lymphomas. Selection of NRE-minus proviruses was not observed in HYB-MTV Δ StuE-induced mammary tumors, even in an animal that developed both a mammary tumor and a thymic lymphoma. These results suggest that the development of lymphomagenic MMTV requires two alterations in the LTR transcriptional regulatory elements, loss of NRE-mediated transcriptional repression and, T-cell enhancer acquisition. Besides TBLV, the LTR transcriptional strengths of two other naturally occurring thymotropic MMTV variants (MA and DL-8) have been tested in T-cell lines. Both of these LTRs were also shown to harbor T-cell enhancers along with deletions within the NRE.

The selection of NRE-minus recombinants in HYB-MTV Δ StuE-induced lymphomas may be driven by the incomplete suppression of NRE activity by the upstream T-cell enhancer as observed in transient transfection assays in T cells. HYB-MTVA Δ E is not subject to this selection pressure since the T-cell enhancer located downstream of the NRE completely masks its repressive activity. Loss of NRE activity and T-cell enhancer acquisition would allow increased viral

expression in T cells resulting in a higher probability of oncogenic insertional mutagenesis. The viral T-cell enhancer also may be critical for transcriptional upregulation of appropriate cellular oncogenes resulting in lymphomagenesis. The composition of viral enhancers has been correlated with altered patterns of proviral integrations near cellular oncogenes [321]. Furthermore, the lymphomagenic ability of HYB-MTV Δ flE confirms that Sag truncation is dispensable for MMTV-induced lymphomagenesis, although T-cell tumor incidence may be increased.

4.1.6 Presence of NREs and T-cell enhancer loss in mammotropic MMTVs

The predominant proviruses expressed in HYB-MTV Δ flE and HYB-MTV Δ StuE-induced mammary tumors lacked the T-cell enhancer while harboring the NREs. Selection of such variants indicates the incompatibility of the T-cell enhancer in the context of MMTV-induced mammary tumorigenesis and might also explain the lower incidence of mammary tumor induction by HYB-MTV Δ StuE and HYB-MTV Δ flE viruses. The T-cell enhancer might negatively affect MMTV LTR-driven transcription in mammary cells (as suggested by transient transfection assays), thus slowing viral replication and oncogenic insertional mutagenesis. The T-cell enhancer could also antagonize MMTV-induced mammary cell-specific oncogene activation required for mammary tumorigenesis.

Interestingly, the predominant proviruses detected in HYB-MTV Δ NRE-induced mammary tumor were recombinants that had acquired the NRE sequences. This observation suggests that the NREs play a positive role in MMTV-induced mammary tumorigenesis by enhancing viral transcription in differentiated

mammary cells or through oncogene activation. The low tumor incidence of the HYB-MTV Δ NRE virus might be due to the requirement for such recombinants.

4.1.7 Summary and conclusions

In conclusion, these studies demonstrated that LTR *cis*-acting transcriptional elements are the critical determinants of MMTV-induced tumorigenesis. Viral transcriptional elements determine the level of viral expression in specific cell types and their potential to activate the appropriate cellular oncogenes. Altered patterns of tumorigenesis resulting from changes in the composition of transcriptional elements in viral LTRs has been extensively reported for murine and feline leukemia viruses, e.g., SL3-3 and FeLV [322, 411]. However, these studies are unique in altering disease specificity between epithelial and lymphoid cell types.

4.1.8 Scope of further studies

The contribution of individual transcription factors in determining MMTV-induced disease remains unclear. However, in view of the rapid selection of recombinants that occurs during *in vivo* experiments, unambiguous results would be difficult to achieve. Future experiments could attempt to target MMTV oncogenesis using tissue-specific enhancers in a promoter-proximal location within the viral LTR. For example, insertion of the immunoglobulin B-cell enhancer upstream of the MMTV promoter may result in recombinant viruses that induce B-cell lymphomas. Such recombinant viruses may be useful in determining novel tissue-specific oncogenes and tumor suppressors.

4.2 An Endogenous *Mtv*-Free BALB/cJ Congenic Mouse Strain is Resistant to MMTV, TBLV and *Vibrio cholerae*.

4.2.1 A novel mode of resistance to MMTV

These studies describe a novel mechanism of resistance to exogenous MMTV(C3H)-induced mammary tumorigenesis in a BALB/cJ congenic mouse strain (BALB/*Mtv*-null) that lacks the three endogenous *Mtvs* resident in the BALB/cJ germline [65, 372]. An immune response to MMTV(C3H) Sag was also negligible in the BALB/*Mtv*-null mice. Deletion of Sag-reactive T cells was not detectable in adult BALB/*Mtv*-null mice inoculated intraperitoneally with XC/HYB-MTV cells, whereas a very low-grade deletion of Sag-reactive cells was observed in mice infected by milk-borne MMTV. The low-grade T-cell deletion in neonatally infected BALB/*Mtv*-null mice might be due to maternal Sag-presenting B cells introduced via milk [16] since non-self MHC class II molecules can also induce an immune response to MMTV Sag [416]. This explanation is consistent with the low-grade deletion observed in the CD4+V β 3+ T-cell subset that is reactive to the endogenous *Mtv*6, but not to the MMTV(C3H) Sag, present in the C3H/HeN strain (Figure 3.16B, left panel).

4.2.2 Role of MMTV Sag in the viral life cycle

Despite impaired Sag-mediated viral amplification, the BALB/*Mtv*-null mice were susceptible to MMTV infection. Viral transcripts were detected by RT-PCR analysis in various mouse organs, including the mammary glands and

spleen. A number of studies have suggested that Sag-induced interaction of T and B cells is critical for a robust infection by exogenous MMTV, leading to oncogenesis. Transgenic mice expressing high levels of MMTV(C3H) Sag delete their Sag-cognate $V\beta 14+$ T cells and fail to be infected by exogenous, milk-borne MMTV(C3H) due to the absence of Sag-reactive T cells [157]. A similar phenotype has been reported in TCR $V\beta 8.2$ transgenic mice that are resistant to MMTV(SW) whose Sag does not recognize TCR $V\beta 8.2$ [184]. Milk-borne MMTV also fails to infect B-cell-deficient mice as determined by the absence of Sag-mediated T-cell deletion and proviral integrations in the spleen and mammary glands [40].

Knock-out mice deficient in cell surface molecules involved in T-B cellular interaction also fail to efficiently propagate milk-borne MMTV. CD40L^{-/-} mice infected by milk-borne MMTV show rare proviral integrations in their spleen and lack Sag-mediated T-cell deletion [76]. Similarly, mice deficient in CD28/CTLA4-B7 interactions show minimal Sag-mediated peripheral T-cell deletion and limited viral dissemination [73], whereas MHC class II deficient mice infected by milk-borne MMTV(C3H) show drastically reduced viral spread from the gut to the mammary gland [41].

4.2.3 Resistance of BALB/*Mtv*-null mice to TBLV

The resistance of BALB/*Mtv*-null mice to MMTV-induced tumorigenesis may be due to the lack of Sag-mediated immune response and poor viral amplification in the lymphoid compartment. However, BALB/*Mtv*-null mice were also resistant to Sag-independent, TBLV-induced lymphomagenesis. TBLV is a

T-cell tropic variant of MMTV that encodes a truncated Sag protein lacking the C-terminal region required for TCR recognition [27, 466]. Lack of TBLV-induced tumorigenesis suggests that the resistance phenomenon of BALB/*Mtv*-null mice is linked to the host immune system at a step that is upstream of the Sag response and common to both MMTV and TBLV infections. The lack of Sag-mediated T-cell deletion appears to be a downstream effect of the limiting step because a direct correlation was observed between the presence/absence of Sag-mediated T-cell deletion and susceptibility/resistance, respectively, to MMTV-induced tumors. These results also demonstrate that resistance of BALB/*Mtv*-null mice to tumor induction is independent of the target organs and tissues.

4.2.4 Correlation of *Mtv* loss with MMTV resistance in BALB/*Mtv*-null mice

Genetic mapping linked the resistance phenomenon of BALB/*Mtv*-null mice to the absence of endogenous *Mtvs* in this strain. BALB/cJ congenic strains containing any one of the three endogenous proviruses (*Mtv*6, 8 or 9) were susceptible to MMTV(C3H) Sag-mediated T-cell deletion and mammary tumorigenesis. The endogenous *Mtv*-free siblings of mice carrying single *Mtvs*, did not undergo MMTV(C3H) Sag-mediated T-cell deletion and also demonstrated a lower mammary tumor incidence. The extent of deletion as well as the tumor incidence in XC/HYB-MTV injected BALB/*Mtv*-positive mice was lower than that of injected BALB/cJ mice; this result may be due to a gene dosage effect since BALB/cJ mice contain two copies each of *Mtv*6, 8, and 9 while the BALB/*Mtv*-SP mice contain one or two copies of a single endogenous provirus.

A combination of two or more endogenous *Mtv* strains may be required to achieve the level of susceptibility observed in BALB/cJ mice. In agreement with this idea, BALB/cJ x BALB/*Mtv*-null F1 mice, which harbor a single copy each of *Mtv*6, 8, and 9, were very similar to BALB/cJ mice in their susceptibility to MMTV-induced tumorigenesis and kinetics of Sag-mediated T-cell deletion. Thus, the presence of endogenous *Mtvs* in BALB/cJ mice renders them susceptible to exogenous MMTV(C3H)-induced tumorigenesis. These observations demonstrate that although endogenous *Mtvs* were proposed to protect against infection by exogenous MMTV strains of similar Sag specificity [157, 184], they can also facilitate tumorigenesis by exogenous MMTVs encoding Sags of alternate TCR V β specificity.

4.2.5 Lack of MMTV-neutralizing antibodies in BALB/*Mtv*-null mice

Another endogenous *Mtv*-free mouse strain derived from a BALB.D2 (inbred, *Mtv*6, 7, 8, 9) x 38CH (wild-derived inbred, *Mtv*-free) cross has been described to be relatively resistant to MMTV infection and cancer development [139]. The resistance phenomenon was attributed to the lack of immune tolerance to MMTV since the neonatal immune system of *Mtv*-free mice develops in the absence of endogenous *Mtv*-encoded proteins [139]. Compared to *Mtv*-expressing mice, higher levels of MMTV-specific antibodies were detected in these *Mtv*-free mice in response to exogenous MMTV infection. Increased neutralizing antibodies were implicated in providing partial protection against chronic MMTV infection, resulting in viral clearance within a few generations [139]. Low titer, MMTV-

specific antibodies also were detected in the sera of about 30% of the BALB/*Mtv*-null mice infected by milk-borne MMTV. These antibodies were specific for MMTV capsid protein and did not react with MMTV Env proteins thus, precluding their role in virus neutralization. The remaining 70% of the MMTV-infected BALB/*Mtv*-null mice did not develop any antibodies against MMTV. Lack of Sag-mediated T-cell deletion in BALB/*Mtv*-null mice also distinguishes them from the BALB.D2-congenic *Mtv*-free strain [139]. Therefore, the resistance of BALB/*Mtv*-null mice to MMTV-induced tumorigenesis is linked to the absence of endogenous *Mtvs* and cannot be attributed to antibody-mediated viral clearance and lack of tolerance to virus infection.

4.2.6 Susceptibility to MMTV in other *Mtv*-free mouse strains

Resistance to tumorigenesis by exogenous MMTV has not been described in other known strains of *Mtv*-free mice. The wild-derived inbred strain Czech II is free of endogenous *Mtvs* but harbors a milk-transmitted exogenous MMTV(Czech II) virus [144]. Czech II mouse colonies display an average mammary tumor incidence of 12% with a latency of 9 to 28 months [144]. The low tumor incidence has been interpreted to be a result of the low virulence of MMTV(Czech II). Infection studies using highly tumorigenic strains of MMTV have not been performed in these mice. An inbred, endogenous *Mtv*-free mouse strain derived from a cross between C58/J and CBA/CaJ strains also has been described [388]. This strain was shown to be susceptible to infection by a newly described exogenous MMTV strain, JYG-MMTV [471]. Viral RNA was detected in the mammary

glands of infected female mice and also in the milk collected from 2-day old pups, tumorigenicity was not assessed.

4.2.7 Cell-mediated immunity and MMTV resistance of BALB/*Mtv*-null mice

The resistance to exogenous MMTV in BALB/*Mtv*-null mice might be mediated by an enhanced cytotoxic T lymphocyte (CTL) response directed at MMTV-infected cells due to the lack of immunologic tolerance provided by endogenous *Mtvs* in BALB/cJ mice. MMTV Env-derived peptides have been demonstrated to activate peptide-specific CTLs that result in reduced tumor growth [455]. A heightened MMTV-specific immune response mediated by CTLs in the BALB/*Mtv*-null mice might also be the basis for complete rejection of the BALB/cJ B-cell lymphoma line, A20, stably expressing HYB-MTV. However, BALB/*Mtv*-null mice develop A20 tumors, which express endogenous *Mtvs*, with a 29% incidence, perhaps reflecting the greater immunogenicity of HYB-MTV encoded proteins. However, BALB/*Mtv6* mice were susceptible to MMTV-induced tumors, despite the incomplete nature of the *Mtv6* provirus. *Mtv6* has a 6241 bp deletion encompassing most of the *gag* and *env* genes and the entire *pol* gene [78] and this deletion removes most of the immunodominant CTL epitopes of both Gag and Env [153]. Therefore, BALB/*Mtv6* mice would not be tolerant to these epitopes on exogenous MMTV proteins. Preliminary experiments comparing the anti-MMTV CTL responses of BALB/cJ and BALB/*Mtv*-null mice in *ex-vivo* and *in-vivo* assays failed to detect >2% specific lysis in either case (data not shown).

4.2.8 Effect of MMTV Sag on immune response against pathogens

Sags encoded by endogenous *Mtvs* alter the immune repertoire of mice by influencing both positive and negative selection of T cells [388]. Unlike BALB/*Mtv*-null mice, BALB/cJ mice demonstrate almost complete deletion of V β 3, 5, 7 and 12+ T cells from their periphery while harboring a compensatory increase in other TCR V β subsets like V β 14+ T cells [the subset recognized by MMTV(C3H) Sag]. This alteration of T-cell subsets might influence the tumorigenesis of exogenous MMTVs, even for those encoding Sags with different TCR specificities than endogenous *Mtv* Sags.

The Sag-mediated alteration of the T-cell repertoire has been shown to influence the susceptibility or resistance to various infections. In inbred mouse strains bearing the H-2^k MHC haplotype, susceptibility to polyomavirus-induced tumors has been linked to the presence of endogenous *Mtv7* [255]. The *Mtv7*-encoded Sag mediates the deletion of V β 6+ T cells that are essential for mounting a robust anti-tumor CTL response on an H-2^k background. Similarly, *Mtv7* Sag-mediated deletion of TCR V β 8.1+ T cells has been shown to confer resistance to cerebral malaria induced by *Plasmodium berghei* [167]. Sag-mediated deletion of TCR V β 4+ T cells by MMTV(SIM) renders BALB/cJ mice resistant to *Leishmania major* infection [240]. V β 4+ and V β 8+ CD4+ T cells produce an early burst of IL4 in response to *L. major* that induces a Th2 response and susceptibility to infection in BALB/cJ mice. Lastly, resistance to *Plasmodium yoelii* in the BALB/c strain has been linked to the presence of the endogenous *Mtv7* locus [419].

4.2.9 Role of endogenous *Mtvs* during exogenous MMTV infection

Endogenous *Mtv*-encoded protein(s) might cooperate with exogenous MMTV in facilitating (i) efficient infection of specific cell types, for instance DCs and B cells, and/or (ii) appropriate cellular interactions and/or activation and proliferation, e.g., DC-mediated T-cell priming. This cooperation would allow a Sag-mediated immune response, leading to viral spread and tumor induction. However, the role of DCs and B cells in TBLV-induced tumors is unknown. *Ex-vivo* bromodeoxyuridine (BrdU) incorporation assays did not reveal differences in the percentage of cycling cells in the draining lymph nodes following foot-pad inoculation of MMTV (data not shown). Interestingly, milk-borne MMTV(C3H) infection has been demonstrated to induce endogenous *Mtv sag* gene expression in BALB/cJ and C3H/HeN mice [469]. Ten to 30 fold induction of endogenous *Mtv sag* mRNA was observed in the gut-associated lymphoid cells, spleen and thymus of these mice within 24 hr post-infection. This induction was specific for the spliced *sag* transcript since no increase was observed in the total endogenous *Mtv* RNA.

Endogenous *Mtv* Sags might have an additional function in the pathogenesis of an exogenous MMTV. Sag also is the only viral protein that is known to be expressed by the defective *Mtv6* provirus [78] and, in the absence of Rem coding potential, the export of the truncated *gag* mRNA would be very inefficient in BALB/*Mtv6* mice. *Mtv6* also lacks the P_{env} promoter and, hence, the major transcript produced from *Mtv6* is the *sag* mRNA from the standard LTR promoter. This transcript could potentially encode novel proteins by the use of alternate start codons GUG and CUG. The possibility that these novel protein(s) influence

exogenous MMTV pathogenicity remains to be exhaustively tested. RT-PCR analyses of a BALB/c derived cell line (A20) as well as tissue RNA obtained from BALB/*Mtv6* spleen, lymph nodes, and thymus did not detect any novel mRNAs being expressed from the endogenous *Mtv6* locus (data not shown). This observation does not rule out a cell-type specific or infection-triggered expression of novel mRNAs from endogenous *Mtvs*. The *Mtv6* locus was chosen for this analysis because BALB/*Mtv6* mice are susceptible to both MMTV and *V. cholerae*. The truncated endogenous *Mtv6* locus also would facilitate detection of novel mRNAs due to the absence of *gag*, *pol*, *env*, and *rem* mRNAs.

4.2.10 Resistance of BALB/*Mtv*-null mice to *V. cholerae*

Besides MMTV and TBLV, the BALB/*Mtv*-null mice show decreased mortality following infection with *V. cholerae*. Interestingly, the increased resistance to *V. cholerae* correlates with the absence of endogenous *Mtvs* despite a vast difference in the pathogenicity and life cycles of MMTV and *V. cholerae*. A potential connection between the presence of endogenous *Mtvs* and susceptibility to *V. cholerae* might be provided by Sag. The T-cell repertoire of BALB/*Mtv*-null mice, generated in the absence of endogenous *Mtv*-encoded Sag, might present an altered immune response, perhaps involving heightened inflammation or enhanced humoral immunity, which might play a role in clearing the *V. cholerae* infection more effectively. However, adaptive immune responses would not be significantly activated during the 48 hr assay for LD₅₀ determination following a primary bacterial infection.

The innate immune responses would form the primary defense against *V. cholerae* for the duration of the LD₅₀ assay. An increased inflammatory innate immune response against *V. cholerae* in the BALB/*Mtv*-null mice compared to the BALB/cJ and BALB/*Mtv*-SP mice might provide a greater resistance to the bacterial infection, thus resulting in a higher LD₅₀. The influence of endogenous *Mtvs* and *Mtv*-encoded Sags on the mouse innate immune response remains to be elucidated. However, exogenous MMTVs and MuLVs have been shown to activate TLR4 [64, 359]. An endogenous *Mtv*-encoded product may enhance CT activity, thus aggravating the cholera symptoms.

4.2.11 Summary and conclusions

In conclusion, a novel mode of resistance to MMTV- and TBLV-induced tumorigenesis and *V. cholerae*-induced mortality in a BALB/cJ congenic mouse strain (BALB/*Mtv*-null) lacking endogenous *Mtvs* has been described. Both resistance traits genetically correlate with the absence of endogenous *Mtvs* in the BALB/*Mtv*-null mice and occur during early pathogen interaction with the immune system. Endogenous *Mtv*-encoded Sag is a primary candidate in mediating susceptibility to pathogens because Sag is the only protein known to be expressed from the endogenous *Mtv6* locus, which provides susceptibility to MMTV and *V. cholerae*. Sag might function by modulating the response or interaction of the mouse immune system towards pathogens, resulting in the manifestation of the multi-pathogen resistance phenomenon. However, a critical role of a novel *Mtv*-encoded protein in providing pathogen susceptibility cannot be excluded.

4.2.12 Scope of further studies

Future experiments would address the role of CD8⁺ cytotoxic T cells in determining MMTV resistance by infection of BALB/*Mtv*-null mice on a CD8-null background. Transgenic mice expressing single MMTV cDNAs would be used for direct assessment of the contribution of endogenous *Mtv*-encoded proteins in determining the pathogen resistance of BALB/*Mtv*-null mice. Comparison of Th1 (IFN γ) versus Th2 (IL10) cytokine profiles of lymphocytes harvested from MMTV-infected BALB/cJ, BALB/*Mtv*-null, and BALB/*Mtv*-SP mice would determine the relation between endogenous *Mtvs*, the type of immune response and susceptibility to pathogens. The response of the BALB/*Mtv*-null mice to purified cholera toxin also would be compared to the responses of the BALB/cJ and the BALB/*Mtv*-single positive mice. Assessment of the susceptibility of BALB/*Mtv*-null mice to other pathogens, in particular *Salmonella typhimurium* and ectromelia virus, would help characterize the resistance phenomenon and also determine if this mouse strain is resistant to a wider spectrum of mouse pathogens.

Appendix

List of abbreviations used:

ALV	avian leukemia virus
APC	antigen presenting cell
APE1	apurinic/apyrimidinic endonuclease 1
APOBEC	apolipoprotein B mRNA editing catalytic subunit
ARM	arginine-rich motif
ASLV	avian sarcoma-leukosis virus
BAF	barrier-to-autointegration factor
BCP	1-bromo-3-chloropropane
BrdU	bromodeoxyuridine
Bright	B-cell regulator of IgH transcription
CA	capsid
cAMP	cyclic adenosine monophosphate
CDP	CCAAT displacement protein
CDR1	complementarity determining region 1
CFU	colony forming units
CIIPBM	class II peptide binding motif
CIS	common integration site
CT	cholera toxin
CTF	CCAAT transcription factor
CTL	cytotoxic T lymphocyte
CTRS	cytoplasmic targeting-retention signal
DC	dendritic cell
DEPC	diethyl pyrocarbonate
DEX	dexamethasone
DLS	dimer linkage sequence

DMBA	7,12-dimethylbenz (α)-anthracene
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DU	deoxyuridine triphosphatase
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
ELISA	enzyme-linked immunosorbent assay
E μ	immunoglobulin heavy chain intronic enhancer
Env	envelope
ER	endoplasmic reticulum
FACS	fluorescence-activating cell sorting
Fgf	fibroblast growth factor
FITC	fluorescein isothiocyanate
FoxA	forkhead box A
Fv1,4	Friend virus susceptibility genes
FWB	FACS wash buffer
GABP	GA-binding protein
<i>Gapd</i>	glyceraldehyde-3-phosphate dehydrogenase
Gag	group-specific antigen
GC	germinal center
GR	glucocorticoid receptor
GRE	GR-binding element
GRF	general regulatory factor
HERV-K	human endogenous retrovirus-K
HIV	human immunodeficiency virus
HMG	high mobility group
HRE	hormone-responsive element
HRP	horseradish peroxidase
HSV	herpes simplex virus
HTLV-1	human T-cell leukemia virus-1
HV4	hypervariable region 4
IAP	intracytoplasmic A particle
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
IL2R	interleukin 2 receptor

IN	integrase
ISBP	initiation site-binding protein
ITAM	immunoreceptor tyrosine-based activation motif
LD ₅₀	lethal dose 50
LDV	lactate dehydrogenase elevating virus
LI	liver
LTR	long terminal repeat
LUC	luciferase
Lv1	lentivirus restriction factor 1
MA	matrix
MAb	monoclonal antibody
MAF	mammary cell activating factor
MAR	matrix attachment region
MEM	mammary-specific enhancer of MMTV
META	MMTV <i>env</i> transcriptional activator
MG	mammary gland
MGE	mammary gland enhancer
MGF	mammary gland factor
MHC	major histocompatibility
ILs	mixed-lymphocyte stimulating
MLV	murine leukemia virus
M-MLV	Moloney murine leukemia virus
MMTV	mouse mammary tumor virus
MT	mammary tumor
MTVR	MMTV receptor
MVB	multivesicular body
NC	nucleocapsid
NES	nuclear export signal
NF1	nuclear factor 1
NLS	nuclear localization signal
NO	nitric oxide
NRE	negative regulatory element
Oct-1/OTF-1	octamer transcription factor
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBS	primer binding site
PCR	polymerase chain reaction

PE	phycoerythrin
PIC	preintegration complex
PMSF	phenylmethylsulfonyl fluoride
Pol	polymerase (reverse transcriptase and integrase)
PPT	polypurine tract
PR/Pro	protease
PRL	prolactin
R	direct repeat
RCS	reticulum cell sarcoma
Rec	regulator of expression encoded by corf
Ref1	restriction factor 1
Rem	regulator of export of MMTV mRNAs
Rev	regulator of virion expression
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
Sag	superantigen
SAR	scaffold attachment region
SATB1	special AT-rich-sequence binding protein 1
SDS	sodium dodecyl sulphate
SG	salivary gland
SN	spleen
SPSS	Statistical Package for Social Sciences
-sssDNA	minus-strand strong stop DNA
+sssDNA	plus-strand strong stop DNA
STAT5	signal transducer and activator of transcription 5
SU	surface envelope protein
TBLV	type B leukemogenic virus
TBP	TATA-binding protein
TCR	T-cell receptor
TEF-1	transcriptional enhancer factor 1
Tfr1	transferrin receptor 1
TGF β	transforming growth factor β
TK	thymidine kinase
TL	thymic lymphoma
TLR4	toll-like receptor 4
TM	transmembrane envelope protein

TNF α	tumor necrosis factor α
U3	3' unique region
U5	5' unique region
WCL	whole cell lysate
Wnt	<i>wingless</i> -related MMTV integration site
ZAP	zinc finger antiviral protein

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